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CONTENTS OF VOLUME 43

No. 1, January, 1956

PAGE

ASO Yoshiro and AKAMATSU Shigeru. Activation and inhibition of phosphomonoesterase by cyanide and zinc ions... 1

MORITA Yoshikatsu. β-Xylosidase and its specificity 7

TSUSHIMA Keizoo and KAWAI Masami. Interrelation between the function of heme-proteins and the structural modifications of their protein parts. V. Spectroscopic and electrophoretic investigations on the reaction of methemoglobin with sodium dodecylsulfate 13

SATO Tokuro, FUKUYAMA Tomitaro, YAMADA Mieko and SUZUKI Taeko. Metabolism of anthraquinone. I. Isolation of 2-hydroxyanthraquinone from the urine of rats 21

SATO Tokuro, YAMADA Mieko, SUZUKI Taeko, FUKUYAMA Tomitaro and YOSHIKAWA Haruhisa. Conjugation of S^{35}-sulfate with phenolic compounds. II. On the sulfate conjugate of 1,2-dihydroxynaphthalene in the urine of rats dosed with naphthalene 25

FUKUI Toshio and NIKUNI Ziro. Degradation of starch in the endosperms of rice seeds during germination 33

YAMAKAWA Tamio, MATSUMOTO Makoto, SUZUKI Shizue and IIDA Tsuyoshi. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. VI. Sphingolipids of erythrocytes with respect to blood group activities 41

MATSUMOTO Makoto. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. VII. Studies on chondrosamine-containing glycolipid and sphingomyelin of hog blood stroma.... 53

YAMAKAWA Tamio, MATSUMOTO Makoto and SUZUKI Shizue. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. VIII. The nature of hexosamine and fatty acids of blood cells sphingomyelin 63

UMEBACHI Yoshishige and TAKAHASHI Humio. Kynurenine in the wings of the *Papilionid* butterflies 73

HAMAGUCHI Kozo. Studies on protein denaturation by surface chemical method. III. The interaction of urea with lysozyme monolayer 83

YAGI Kunio, MATSUOKA Yoshitaka, KUYAMA Shinpei and TADA Mitsuhiko. Preparation of flavin adenine dinucleotide from *Eremothecium ashbyii* 93

LETTERS TO THE EDITORS

SHIMURA Kensuke, FUKAI Hironobu, SATO Jin and SAEKI Reiko. Non-uniform labeling of the silk fibroin synthesized *in vivo*... 101

ANAN, F. K. and MATSUMURA Yasuo. On the peptic modification of catalase. 103

SEKUZU Ichiro and OKUNUKI Kazuo. Purification and some properties of cytochrome *b* from ox heart muscle 107

No. 2, March, 1956

PAGE

KAWAKITA Yukio. Distribution of lipids in the central nervous system of some vertebrates ... 111

NAKATSU Seiichiro. Characterization of a sucrase in Takadiastase ... 119

ICHIHARA Katashi, IKEDA Shigehiko and SAKAMOTO Yukiya. On the formation of gentisic acid from homogentisic acid by the liver extract ... 129

FUKUDA Toshifumi. Biochemical studies on the formation of the silkprotein. III. The conversion of C^{14}-labeled phenylalanine to tryrosine in the silkworm larva (*Bombyx Mori*) ... 137

NOMURA Masayasu, MARUO Bunji and AKABORI Shiro. Studies on amylase formation by *Bacillus subtilis*. I. Effect of high concentration of polyethylene glycol on amylase formation by *Bacillus subtilis* ... 143

EGAMI Fujio and YAGI Kunio. Biochemical studies on "coenzyme sulfate analogues". I. Effect of adenosine-monosulfate and riboflavin-monosulfate on D-amino acid oxidase ... 153

KAZIRO Koozoo, KIKUCHI Gorō and KAWAI Masami. Reaction of verdohemoglobin formation *in vitro*, with special reference to its quantitative treatment ... 161

KIMURA Tokuji and SASAKAWA Taiji. Lipide metabolism of *Mycobacterium tuberculosis avium*. I. Enzymatic synthesis of hydroxamic acid from fatty acid and its specificity ... 175

NAGAMATSU Atsuo. The change of *N*-substituted amino acids in the animal body. XIV. The fate of C^{14}-methyl group attatched to nitrogen of amino acid ... 185

FUJIWARA Teruko. Studies on chromoproteins in Japanese Nori (*Porphyra tenera*). II. Amino acid compositions of phycoerythrin and phycocyanin ... 195

TAKANO Katsuo. Acceptor relations in the enzymatic transfer of β-galactosyl groups ... 205

SHIMURA Kensuke, SATO Jin, SUTO Seiichi and KIKUCHI Aiko. Amino acid composition of ribonucleoprotein of silkgland ... 217

TAKAHASHI Hajime, TANIGUCHI Shigehiko and EGAMI Fujio. Nitrate reduction in aerobic bacteria and that in *Escherichia coli* coupled to phosphorylation ... 223

TSUSHIMA Keizoo. Interrelation between the function of heme-proteins and the structural modifications of their protein parts. VI. Studies on the reactions of modified methemoglobins with cyanide ... 235

KAJITA Akihiko. Interrelation between the function of heme-proteins and the structural modifications of their protein parts. VII. Sulfhydryl group of modified methemoglobin ... 243

LETTERS TO THE EDITORS

NOMURA Masayasu, MARUO Bunji and AKABORI Shiro. Formation of amylase in a cell-free enzyme preparation ... 251

PAGE

No. 3, May, 1956

IKENAKA Tokuji. Studies on Taka-amylase A. V. Carboxy-terminal group of Taka-amylase A 255

HARA Toshiji. Chemical studies on the organ specificity 263

WATANABE Yasushi and SHIMURA Kensuke. Biosynthesis of threonine from homoserine. V. Nature of an intermediary product 283

IWASAKI Hidekazu, MATSUBAYASHI Ryuzo and MORI Takeshi. Studies on denitrification. II. Production of nitric oxide and its utilization in the N-N linkage formation by denitrifying bacteria 295

KURODA Masakiyo. On the components of bull-frog bile (*Rana catesbiana Shaw*). VI. Chemical structure of α- and β-trihydroxyhomocholene... 307

SHUKUYA Ryoiti and SHINODA Michiko. On the kinetics of the human blood cholinesterase. V. The inhibition of acetylcholinesterase and cholinesterase by hydrogen ion and tetraethylammonium bromide 315

MARUYAMA Yoshiharu and NOMURA Masayasu. Metabolism of pyrrolidone carboxylic acid by bacteria 327

YAGI Kunio, MITSUHASHI Susumu and KOJIMA Yasuhiko. Oxidation of lactate by *Corynebacterium diphtheriae* adapted to iron-deficient media ... 337

KOIZUMI Takayoshi, UCHIDA Makio and ICHIHARA Katashi. Studies on histidine deaminase 345

HAMAGUCHI Kozo. Studies on protein denaturation by surface chemical method. IV. On the structure of lysozyme monolayer... 355

KURODA Kaichiro and MISHIRO Yukihiko. Formation of biconcave dicoid form of oil droplet in aqueous protein solution 369

YAMAZAKI Isao, FUJINAGA Kei, TAKEHARA Ichiro and TAKAHASHI Hiroshi. Aerobic oxidation of triose reductone by crystalline turnip peroxidase 377

HONDA Koichiro, OTSUKA Shigeru and SHIMODA Yoshio. Studies on nitroreductase of mammalia. I. Nitro-reducing capacity of the liver and its relation to dehydrogenase systems 387

YAMAGUCHI Kiyoaki. Studies on the absorption of unnatural monosaccharides and their fates after being absorbed 399

LETTERS TO THE EDITORS

YAMAMURA Yuichi, MATUI Kunio and MAEDA Hideo. The presence of pyridine in liporprotein of BCG 417

No. 4, July, 1956

SATO Tokuro, SUZUKI Taeko, FUKUYAMA Tomitaro and YOSHIKAWA Haruhisa. Studies on conjugation of S^{35}-sulfate with phenolic compounds. III. Metabolism of *p*-cresol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in rat liver 413

PAGE

SATO Tokuro, SUZUKI Taeko, FUKUYAMA Tomitaro and YOSHIKAWA Haruhisa. Studies on conjugation of S^{35}-sulfate with phenolic compounds. IV. Metabolism of *o*-cresol, *m*-cresol, salicylaldehyde, salicylic acid, toluene, benzoic acid and related substances in rat liver 421

KOTAKE Yahito, Jr., INOUYE Kaoru and NAKAI Osamu. Effect of tryptophan and anthranilic acid upon adrenalectomized rat. II. Studies on the protein metabolism 431

KOTAKE Yahito, Jr. and NOGAMI Totaro. Studies on xanthurenic acid. XIII. Effect of 5-hydroxyanthranilic acid on the formation and on its conjugating reaction of xanthurenic acid in albino rat 437

OKUNUKI Kazuo, HAGIHARA Bunji, MATSUBARA Hiroshi and NAKAYAMA Takeyoshi. Denaturation and inactivation of enzyme proteins. I. Bacterial proteinase method for the determination of the ratio of denaturation of globular proteins 453

HAGIHARA Bunji, NAKAYAMA Takeyoshi, MATSUBARA Hiroshi and OKUNUKI Kazuo. Denaturation and inactivation of enzyme proteins. II. Denaturation and inactivation of bacterial amylase 469

HAGIHARA Bunji, NAKAYAMA Takeyoshi, MATSUBARA Hiroshi and OKUNUKI Kazuo. Denaturation and inactivation of enzyme proteins. III. Denaturation and inactivation of Taka-α-amylase 483

HAGIHARA Bunji, SHIBATA Takeshi, SEKUZU Ichiro, HATTORI Fumio, NAKAYAMA Takeyoshi, NOZAKI Mitsuhiro, MATSUBARA Hiroshi and OKUNUKI Kazuo. Denaturation and inactivation of enzyme proteins. IV. Denaturation and inactivation of catalase 495

TSUSHIMA Keizoo. Interrelation between the function of heme-proteins and the structural modifications of their protein parts. VIII. Studies on the action of methemoglobin with formaldehyde 509

BITO, Y. The quantitative determination of amino acids by paper chromatography 523

ISHII Shin-ichi. Ion-exchange chromatography of amino acid and peptides. I. A new determination method of basic amino acids on a carboxylic acid resin 531

KAZIRO Koozoo, UCHIMURA Fujiko and KIKUCHI Gorō. Competition of pyridine and cyanide to heme and hematin 539

KITAGAWA Masayasu. Studies on the oxidation mechanism of methyl group 553

SAKOTA Naokazu. On the mechanism of the activation of α-chymotrypsinogen to α-chymotrypsin. IV. The peptide liberation during activation 565

LETTERS TO THE EDITORS

MINAKAMI Shigeki, ISHIKURA Hisayuki and SATAKE Kazuo. Chromato-
of cytochrome c on ion-exchange resin 575

No. 5, September, 1956

PAGE

AKAZAWA Takashi and URITANI Ikuzo. Respiratory increase and phosphorus and nitrogen metabolism in sweet potato infected with *Ceratostomella fimbriata* 579

AKAZAWA Takashi. Metabolic activation of white potato tissue infected with *Ceratostomella fimbriata* 589

AKAZAWA Takashi. Nature of protein synthesis in sweet potato tissue infected with *Ceratostomella fimbriata* 597

ICHIHARA Katashi, ITAGAKI Shigenobu, SUZUKI Yoshitoshi, UCHIDA Makio and SAKAMOTO Yukiya. Studies on the oxidative decomposition of urocanic acid by liver extract. I 603

OTA Shozo, WACHI Tsutomu, UCHIDA Makio, SAKAMOTO Yukiya and ICHIHARA Katashi. Studies on the oxidative decomposition of urocanic acid by *Pseudomonas aeruginosa*. II 611

ICHIHARA Katashi and SAKAMOTO Yukiya. 7-Hydroxyindole derivatives and the acid diazo-reaction 619

SATO Ryo, TAKEMORI Shigeki and EBATA Mituo. Some properties of a bacterial glucokinase 623

YAGI Kunio. Simplified lumiflavin method for the microdetermination of flavin compounds in animal tissues 635

AKAEDA Ikuro. Hexokinase of rabbit testis 645

AKAEDA Ikuro. Comfirmation of glucose-1-phosphate produced by glucokinase of rabbit testis 649

OGATA Kikuo, OGATA Masana, MOCHIZUKI Yoshio and NISHIYAMA Tadamoto The *in vitro* incorporation of C^{14}-glycine into antibody and other protein fractions by popliteal lymph nodes of rabbits following the local injection of crystalline ovalbumin 653

EGAMI Fujio, NAOI Mariko, TADA Mitsuhiko and YAGI Kunio. Biochemical studies on "coenzyme sulfate analogues". II. Effects of riboflavinmonosulfate on bacterial growth 669

NAKATSU Seiichiro. Enzymatic transamidination from canavanine to glycine by hog kidney extracts 675

YAMAWAKI Shigeru. Diaryl pyrophosphatase and FAD pyrophosphatase ... 683

SUZUKI Sakaru. Studies on the incorporation of S^{35}-sulfate into charoninsulfuric acid by *Charonia lampas* 691

SUZUKI Sakaru and ŌGI Ken-ichi. Autoradiographic study on the incorporation of S^{35}-sulfate into mucous gland of *Charonia lampas* 697

ITO Yosoji, TSURUFUJI Susumu, HIRAMATSU Yosio and CHIBA Mikito Physiological chemistry of the hard tissues. I. Dynamics of labeled calcium uptake by the incisor of the rat 701

SABURI Yasuo, HAYAKAWA Shohei, FUJII Takao and AKAEDA Ikuro. Microbiological degradation of bile acids. V. On a principal intermediate in the breakdown of cholic acid by *Streptomyces* 711

PAGE

HAYAKAWA Shohei, SABURI Yasuo, FUJII Takao and SONODA Yoshiya. Microbiological degradation of bile acids. IV. On the structure of the unsaturated C-22 acid transformed from cholic acid by *S. gelaticus* 1164... 723

HAYAKAWA Shohei, SABURI Yasuo, FUJII Takao and SONODA Yoshiya. Microbiological degradation of bile acids. VII. Partial synthesis of 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its derivatives from cholic acid... 731

LETTERS TO THE EDITORS

TITANI Koiti, YOSHIKAWA Hiroshi and SATAKE Kazuo. Half cystine, an additional *N*-terminal group of serum albumin 737

No. 6, November, 1956

AKABORI Shiro, OKADA Yoshimi, FUJIWARA Sadao and SUGAE Ken-ichi. Studies on bacterial amylase. I. Amino acid composition of crystalline bacterial amylase from *B. subtilis* N 741

KONDO Yoichi, KAMEYAMA Tadanori and TAMIYA Nobuo. The decomposition of β-mercaptopyruvic acid by *E. coli* and its extracts 749

TSUSHIMA Keizoo and MIYAJIMA Toshina. Interrelation between the function of heme-proteins and the structural modifications of their protein parts. IX. Perturbation of cytochrome c.... 761

SHIIO Isamu, MARUO Bunji and AKABORI Shiro. Transaminases in a halophilic bacterium No. 101 779

TAZAWA Yasuo und HIROKAWA Toyoyasu. Über Pflanzenproteasen. VI. Die proteolytische Aktivität des Soja-Legumelins 785

ICHIHARA Katashi, SAKAMOTO Yukiya, SATANI Hitoshi, OKADA Naotada, KAKIUCHI Shiro, KOIZUMI Takayoshi and OTA Shozo. Studies on the oxidative decomposition of urocanic acid. III. Isolation of succinic monoureide 797

ICHIHARA Katashi, YOSHIMATSU Hisao and SAKAMOTO Yukiya. Studies on phenol formation. III. Ammonium and potassium ions as the activator of β-tyrosine 803

FURIYA Shin, IZUMITANI Tetsuo and AKAMATSU Shigeru. Consecutive hydrolysis of phosphodiester by phosphodiesterase and phosphomonoesterase 811

ICHIHARA Katashi, SAKAMOTO Yukiya, WADA Hiroshi, OKADA Naotada, ITO Kin-ichi and SHINKAI Toshiro. On the opening and splitting of indole of tryptophan 821

TANAKA Kentaro, MAEKAWA Shizue, HAYASHI Takayoshi and KUROIWA Yoshiro. Biochemical studies on Streptolysin S. I. Effects of alkali-treated ribonucleic acid on the hemolysin formation 827

TANAKA Kentaro, MAEKAWA Shizue, HAYASHI Takayoshi and KUROIWA Yoshiro. Biochemical studies on Streptolysin S. II. Effects of ribonuclease digestion of ribonucleic acid on the hemolysin formation 833

PAGE
NOMURA Masayasu, HOSODA Junko, MARUO Bunji and AKABORI Shiro. Studies on amylase formation by *B. subtilis*. II. Effect of amino acid analogues on amylase formation by *B. subtilis*; an apparent competition between amylase formation and normal cellular protein synthesis 841
SAKURAI Setsuji. Enzymatic preparation of optically active essential amino acids. I. The preparation of L-phenylalanine 851
OKUNUKI Kazuo, MATSUBARA Hiroshi, NISHIMURA Shigehiko, and HAGIHARA Bunji. Specificity of crystalline bacterial proteinase 857
YAMAKAWA Tamio. On the so-called sialic acid of blood cells and serum... 867
INDEX TO AUTHORS i
INDEP TO SUBJECTS ix

The Journal of Biochemistry, Vol. 43, No. 1, 1956

ACTIVATION AND INHIBITION OF PHOSPHOMONOESTERASE BY CYANIDE AND ZINC IONS

By YOSHIRO ASO and SHIGERU AKAMATSU

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(Received for publication, October 20, 1955)

Activation of alkaline apo-phosphomonoesterase in the presence of magnesium ions by amino acids or peptides was interpreted as due to the formation of an enzymatically active complex of the apoenzyme with magnesium and the organic substance (*1–4*). However, later experiments revealed that in the presence of magnesium any reagent capable in general of forming a complex with heavy metals can activate the apo-monoesterase. Our new concept is that alkaline phosphomonoesterase is an apoenzyme-magnesium complex and that the action of amino acids or peptides is attributed to the deprivation of inhibitory heavy metals. The present communication concerns with the effect of cyanide on apo- and holoenzymes.

METHODS

The diluted apo-monoesterase solution was prepared as follows. Ten mg. per cent solution of Alber's phosphomonoesterase powder was dialysed successively against 1 per cent borax and then 2 per cent sodium hydrogen carbonate solution, each for one day, and then was diluted before each experiment to ten times as much volume with water. Sodium *p*-nitrophenyl phosphate was used as the substrate. A mixture of 1 ml. diluted apoenzyme, 1 ml. $M/20$ magnesium chloride, 1 ml. $M/800$ histidine, 2 ml. $M/10$ veronal buffer (pH 9.0), and 3 ml. water was preincubated at 37° for ten minutes and to it was added 2 ml. of $M/400$ substrate solution. For the tests of cyanide or zinc addition the volume of water in the above mixture was replaced by the respective salt solution, which had been adjusted to pH 9.0, and when histidine or magnesium was to be omitted from the mixture the volume of water was correspondingly increased.

Dialysed kidney autolysate which was used as the monoesterase solution already active without any addition was prepared as usual and diluted fifty times with water for the experiments. Composition of the test solution was as follows: A mixture of 1 ml. diluted autolysate, 2 ml. $M/10$ veronal buffer (pH 9.0), and 5 ml. water was preincubated for 10 minutes and 2 ml. of $M/400$ substrate solution was added to it. The volume of water in the above mixture varied for the cyanide and zinc tests. Magnesium or histidine addition was omitted in this case. Throughout the experiments total volume was 10 ml.

Liberated *p*-nitrophenol was estimated by introducing at time intervals 2 ml. aliquot of the test solution into 2 ml. of saturated sodium carbonate solution and measuring the intensity of yellow color produced with the Pulfrich's photometer (filter S 43). Increase of optical density was proportional with time up to 60 minutes. Previous deproteinization was omitted since the diluted enzyme solutions used were colorless and poor in protein.

RESULTS

Holoenzyme Inhibition by Cyanide—The activity of alkaline holoenzyme, composed as mentioned above of apoenzyme, histidine, and magnesium was inhibited to various extents by cyanide addition. When the final concentration of the cyanide is expressed in terms of pCN, i.e. negative logarithm of its final concentration, a sigmoid pCN-inhibition curve of the second order is obtained (Fig. 1).

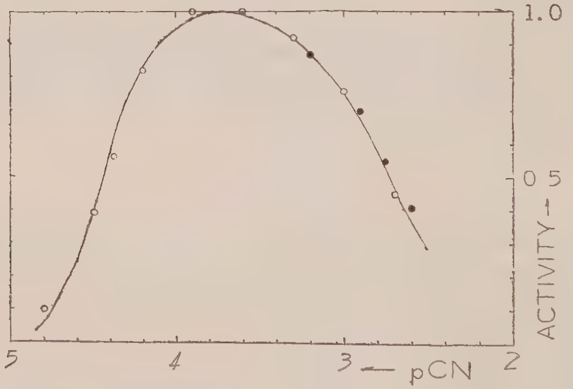

FIG. 1. pCN Activation and inhibition curves.
Conditions of the test are described in the text.
pCN is the negative logarithm of final KCN concentration.

● Inhibition by cyanide of holoenzyme produced by activating the apoenzyme to the maximum extent in the presence of 1.25×10^{-4} *M* histidine and 5×10^{-3} *M* $MgCl_2$.

○ Activation of apoenzyme by cyanide in the presence of 5×10^{-3} *M* $MgCl_2$ and inhibition of thus activated holoenzyme by excess of cyanide.

Apoenzyme Activation by Cyanide and Inactivation of the Holoenzyme thus Formed by Excess of Cyanide—When cyanide was added in increasing amounts in the presence of magnesium to the apoenzyme solution the phosphomonoesterase activity of the solution increased at first and the activation to maximum extent was attained at the final concentrations of cyanide between 2.5×10^{-4} and 1.25×10^{-4} *M*. The extent of activity at the maximum was just the same to that revealed in the case of activation by histidine addition. When the concentrations of cyanide added were made higher than that range, a decrease of monoesterase activity was observed. The form of the pCN-activity curve was very similar to the case of histidine addition to the apoenzyme and was composed of an ascending sigmoid branch of the third order and a descending sigmoid of the second order. Moreover it is remarkable that this descending branch agrees fairly well with the pCN-inhibition curve illustrated above of the apoenzyme activated by histidine to the maximum extent.

Role of Magnesium in the Activation of Apoenzyme by Cyanide—When the magnesium addition is omitted, the formation of the holoenzyme remains within the range of 30 per cent even at the optimal concentration of cyanide (Table I). Magnesium is necessary for the activation by cyanide as well as by histidine (*3*). This activity shown without extra addition of magnesium should be attributed as previously discussed to an unavoidable contamination in the test mixture of a trace of magnesium.

TABLE I

Necessity of Magnesium for Apoenzyme Activation

Composition of enzyme solution tested	Extinction after 60 min.
Apoenzyme	0
Apoenzyme and KCN	0.10
Apoenzyme, KCN, and $MgCl_2$	0.35
Apoenzyme and histidine	0.11
Apoenzyme, histidine, and $MgCl_2$	0.35

The test conditions are described in text. Final concentration: KCN *M*/5000, $MgCl_2$ *M*/200, and histidine *M*/4000.

Effect of Zinc Ion on Phosphomonoesterase—Zinc itself is an inhibitior to the alkaline phosphomonoesterase, since when zinc acetate was added at the final $M/1000$ concentration, either the enzyme in the simply dialysed kidney autolysate or the holoenzyme formed in the presence of magnesium by activating the apoenzyme to the maximum extent by addition of the final $M/4000$ concentration of histidine was inhibited to 30 per cent activity (Table II).

However, when zinc acetate of that amount was added in the presence of potassium cyanide of final $M/250$ concentration, the extent above mentioned of the inhibition became less and a restoration up to nearly 70 per cent activity was observed. That concentration of cyanide is far beyond the optimum ($M/4000$) indicated for the complete activation of the apoenzyme and is within the range where either the monoesterase in the kidney autolysate or the apoenzyme activated to the maximum extent would have been inactivated mostly, if zinc were not present. These results should be explained as due to zinc-cyanide complex formation.

TABLE II

Effect of Zinc Ion upon Holoenzyme Activity

Composition of enzyme solution tested	Activity
Apoenzyme, histidine, and $MgCl_2$	1.00
Apoenzyme histidine, $MgCl_2$, and $Zn(AcO)_2$	0.29
Apoenzyme, KCN, and $MgCl_2$	0.11
Apoenzyme, KCN, $MgCl_2$, and $Zn(AcO)_2$	0.68
Kidney autolysate	1.00
Kidney autolysate and $Zn(AcO)_2$	0.28
Kidney autolysate and KCN	0.19
Kidney autolysate, KCN, and $Zn(AcO)_2$	0.75

Final concentration of additions: Histidine $M/4000$, $MgCl_2$ $M/200$, KCN $M/250$, and $Zn(AcO)_2$ $M/1000$.

It was noticed already (see Table I) that the apoenzyme solution could carry out some holoenzyme action even without any extra addition of magnesium when histidine or potassium cyanide was present at the final concentration of $M/4000$ and $M/5000$, respectively. A result unequivocally attributable to the presence of a trace of magnesium in the test mixture. This residual activity observed in the presence of histidine but without an extra addition of magnesium is not intensified by zinc acetate of final 1×10^{-6} to 2×10^{-5} M concentrations, which are so low that any inhibition of the holoenzyme once formed should never be seen. Zinc is unable to substitute the magnesium action.

DISCUSSION

Activation of the apoenzyme preparation of alkaline phosphomonoesterase by cyanide in the presence of magnesium was observed. The mechanism seems to be the deprivation of some contaminating heavy metals. According to our new theory the alkaline monoesterase is composed of an apoenzyme and magnesium, and inhibition caused by cyanide or amino acids at their high concentrations seems rather to be attributed to their combination with enzyme-substrate complex. Data will be reported in the near future.

In regard to the action of cyanide on alkaline phosphomonoesterase some reports have been communicated. Albers (*5*) assumed that disulfide group of the enzyme protein might be the active center of the monoesterase and he attributed the inhibition by cyanide to the reduction of the disulfide to an inactive sulfhydryl form. However, the role of disulfide group has not been explained. The two metal theory of Cloetens (*6*) says that alkaline monoesterase contains zinc and magnesium as the activating factors and the inhibitory action of cyanide is no more than the deprivation of zinc ion through complex salt formation. Our present experiment indicates rather that zinc is not an essential constituent, though the enzyme preparation may be contaminated by it as well as by other heavy metals. According to a recent report of Sadasivan (*7*) the cyanide-inhibited phosphomonoesterase regains the activity on the addition of zinc, as it was confirmed by the present experiment. However, his assumption that zinc is an essential constituent of the monoesterase would not be simply deduced from such an experiment. Zinc ion is rather inhibitory as well as other heavy metal ions to alkaline monoesterase. On the other hand, Hove *et al.* (*8*) reported that zinc enhanced the action of undialysed intestine phosphatase. This would probably be due to the removal of inorganic phosphate produced during autolysis and by itself capable of competitively inhibiting the monoesterase, since the same authors proved that the dialysed bone, kidney, and intestine monoesterase were inhibited by zinc addition.

SUMMARY

Apoenzyme of alkaline phosphomonoesterase is activated in the presence of magnesium by cyanide of definite concentrations ranging between *M*/8000 and *M*/4000. It is similar to the cases previously observed with histidine, other amino acids, or the related compounds. The mechanism of these activations is now assumed to be the deprivation of inhibitory effect of some contaminating heavy metals. On the other hand, cyanide at higher concentrations than *M*/2000 inhibits the active enzyme produced by mixing apoenzyme, magnesium, and the optimal amounts of either histidine or cyanide. It is also similar to the inactivation of alkaline monoesterase by addition of an excessive amount of histidine or other amino acids. The activities thus inhibited at high concentration of cyanide can be regained by addition of zinc salt due to zinc-cyanide complex formation. However, zinc itself is not essential to the monoesterase action and rather inhibitory to the enzyme, may it be the holoenzyme contained in the dialysed kidney autolysate or that formed by activation of the apoenzyme in the presence of magnesium by histidine addition.

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β-XYLOSIDASE AND ITS SPECIFICITY

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Our studies on β-hexosidase (*1*, *2*) have resulted in a conclusion that β-glucosidase and β-galactosidease are different enzymes, the specificity of which is determined by the configuration of the sugar moiety. The union of the enzyme with the specific substrate takes place at two points, hexose moiety and β-hexoside linkage.

However, there exist in nature for each enzyme two isodynamic enzymes of Taka and emulsin types, which can be differentiated according to the mode of formation of enzyme-substrate complex. The enzyme of Taka type forms at first an intermediate complex with the substrate at its sugar residue of specific configuration and then combination with β-heteroside linkage takes place within the enzyme-substrate complex. Hydrolysis of *p*-nitrophenyl β-glucoside by β-glucosidase of Taka type is inhibited by glucose, gluconate, phenyl β-glucoside and gluconolactone, but not at all by galactose and its corresponding derivatives, which are, however, the specific inhibitors to the β-galactosidase of Taka type. On the other hand since the enzyme of emulsin type unites at first with β-hexoside linkage of the substrate and then with the hexose moiety, the inhibition by phenyl β-glucoside, phenyl β-galactoside, gluconolactone, and galactonolactone is common to the hydrolysis of *p*-nitrophenyl β-glucoside and β-galactoside by respective enzymes of emulsin type, while glucose, galactose, gluconate, and galactonate show no inhibitory action upon both enzymes of this type. In the present investigation the specificity of β-xylosidase was studied, since in *p*-nitrophenyl β-xyloside the terminal alcohol group of β-glucoside is replaced by a hydrogen atom. Inhibition of glucuronate and glucosaccharolactone upon β-glucosidase and β-xylosidase activity was also tested.

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* The result of the experiment was communicated in Japanese two years ago (*3*).

EXPERIMENTAL

p-Nitrophenyl β-xyloside (m.p. 144°) and phenyl β-xyloside (m.p. 178°) were prepared from their triacetyl derivatives, and mucic acid and potassium hydrogen glucosaccharate according to Fischer (*4*). Glucosaccharolactone prepared by the method of Kiliani (*5*) was washed with acetone. Xylonolactone prepared according to Clowes-Tollens (*6*) from cadmium xylonate bromide (*7*) was syrupy and could not be obtained in crystalline form.

p-Nitrophenyl β-xyloside, glucoside, and galactoside were used as substrates. Preparation of enzyme solutions was made as described previously (*2*). Composition of test solution was as follows: 10 ml. of *M*/1000 substrate solution, 5 ml. of buffer solution, 3 ml. of water, 2 ml. of enzyme solution, and some drops of toluene. In the inhibition experiment, the substance to be tested was dissolved in a mixture of buffer and water (5:3) at the concentration of *M*/8, *M*/80 and *M*/800 and each 8 ml. aliquot was used. The final concentrations of the inhibitory substance in the reaction mixtures were therefore *M*/20, *M*/200, and *M*/2000, or 100-, 10- fold over and equal to the substrate concentration, respectively. The liberated *p*-nitrophenol was estimated photometrically in the alkalized solution as usual.

Optimum pH of β-xyloside hydrolysis by Takadiastase was 5 and that by emulsin 6, as in the case of β-glucoside hydrolysis. The enzyme activities upon both substrates were compared at their respective optimum. It was found that Takadiastase hydrolysed both substrates approximately at the same rate as reported by Miwa (*8*), whereas in the case of emulsin the hydrolysis of β-glucoside proceeded faster than that of β-xyloside, as previously shown by Helferich (*9*).

The question whether *p*-nitrophenyl β-xyloside could be hydrolysed by β-glucosidase and β-galactosidase was tested. The solution of β-glucosidase of Taka type was prepared from seed of *Andropogen Sorghum* (Morokoshi in Japanese). β-Galactosidase of Taka type is contained in the dialysed autolysate of pig spleen. Suspension of *Proteus vulgaris* harvested on agar broth containing 1 per cent salicine was used as β-glucosidase of emulsin type and the suspension of *Escherichia coli communis* as β-galactosidase of emulsin type. The results are indicated in Table I.

This result indicates that β-xylosidase is an enzyme different from β-glucosidase and β-galactosidase. A slight hydrolysis of β-xyloside by Morokoshi would indicate that β-xylosidase of Taka-type is present in nature often together with β-glucosidase.

Taka β-glucosidase solution free from β-xylosidase activity can be prepared in the following manner, whereas the isolation of β-xylosidase from Takadiastase was without success. To 8 ml. of 2 per cent Takadiastase solution was added 2 ml. of *M*/10 disodium hydrogen phosphate and after adjusting to pH 9 by the addition of dilute ammonia the mixture was shaken with 0.5 g. of alumina for 30 minutes and then centrifuged. The supernatant solution was treated once more with alumina and neutralized with dilute hydrochloric acid. This solution hydrolysed 91 per cent of *p*-nitrophenyl β-glucoside but not β-xyloside. A similar β-glucosidase solution could be obtained when that Takadiastase solution buffered at pH 9 was left to stand for

TABLE I

β-Xyloside Hydolysis

Substrate	Taka type		Emulsin type	
	β-Glucosidase (*Andropogen sorghum*)	β-Galactosidase (pig spleen)	β-Glucosidase (*Proteus vulgaris*)	β-Galactosidase (*Escherichia coli communis*)
Nitrophenyl β-glucoside	⧺	—	⧻	—
Nitrophenyl β-galactoside	—	⧻	—	⧻
Nitrophenyl β-xyloside	+	—	—	—

⧻ rapid hydrolysis
+ slow hydrolysis
— no hydrolysis

2 hours and neutralized. This enzyme solution hydrolysed in 2 hours β-glucoside to 51 per cent but not β-xyloside at all.

Inhibition test with β-xylosidase was carried out just as reported in the case of β-glucosidase and β-galactosidase inhibition (*2*). First, the inhibitory effect of glucose, xylose, and their β-phenyl derivatives to *p*-nitrophenyl β-glucoside and β-xyloside hydrolysis by Takadiastase and emulsin was compared. The results are summarized in Table II.

TABLE II

Extent of β-Heterosidase Inhibition

	Takadiastase		Emulsin	
Substrate / Inhibitor	NP*-β-glucoside	NP-β-xyloside	NP-β-glucoside	NP-β-xyloside
Glucose	(⧺)	(—)	(—)	(—)
Xylose	(—)	(⧺)	(—)	(—)
Phenyl β-glucoside	(⧻)	(—)	(+)	(+)
Phenyl β-xyloside	(+)	(⧻)	(+)	(+)

NP*—*p*-nitrophenyl; (⧻) strong inhibition, (—) no inhibition.

Inhibitory effect of gluconate, xylonate, xylonolactone, glucuronate, glucosaccharolactone and mucate was then tested at pH 6.4. Of these sugar derivatives only xylonolactone inhibited β-glucosidase, β-galactosidase, and β-xylosidase of emulsin preparation and β-xylosidase of Takadiastase, while other two enzymes of Takadiastase remained uneffected.

These experiments were carried out at pH 6.4, since at pH 5 xylonic acid may be converted to its lactone, which would behave against β-heterosidase in a manner different from xylonate. This precaution was taken because, contrary to our findings, Takano (*10*) reported that gluconic acid was inhibitory to β-glucoside and β-galactoside hydrolysis by emulsin. His experiment had been performed at pH 5. Therefore, the action of Takadiastase and emulsin upon β-hexosides was compared in the absence and presence of gluconic acid at pH 5 and 6 (Table III). At pH 5 hydrolysis of three β-heterosides by emulsin was slightly inhibited by gluconic acid.

This inhibitory effect of gluconic acid was intensified further as the acidity of the medium increased owing to the acceleration of the spontaneous lactone formation (Table IV).

Table III

Inhibition by Gluconic Acid

	Takadiastase		Emulsin	
Substrate	pH 5	pH 6	pH 5	pH 6
Np-β-glucoside	(+++)	(++)	(+)	(−)
NP-β-galactoside	(−)	(−)	(+)	(−)
NP-β-xyloside	(−)	(−)	(+)	(−)

NP—*p*-nitrophenyl; (+++) strong inhibition, (—) no inhibition.

Table IV

p-Nitrophenyl β-Glucoside Hydrolysis by Emulsin in the Presence of Gluconate

pH	4	4.5	5	5.8	6	6.2	6.4	7	7.5
M/20 Gluconate	3	5	9	27	31	32	28	22	17
None (Control)	23	29	32	39	38	34	28	22	16

Final concentration of substrate, *M*/2000; emulsin 0.1 per cent; incubation 2 hours. The numerical values represent hydrolysis in per cent.

DISCUSSION

As expected from our theory indicated at the introduction of this paper, hydrolysis of *p*-nitrophenyl β-xyloside by β-xylosidase of Taka type which unites at first with the xylose moiety of the substrate is inhibited by the addition of xylose and phenyl β-xyloside, but not at all by glucose or phenyl β-glucoside, while that by β-xylosidase of emulsin type is inhibited by phenyl β-xyloside and also by β-glucoside, but not by xylose or glucose, since the enzymes of this type prefers the heteroside linkage for the first combination with the substrates.

Inhibiting action of phenyl β-glucoside which has in the molecule the specific glucose residue and a general heteroside linkage, was reported previously (*2*). That glucoside was an inhibiting agent to β-glucosidase of Taka type and to β-glucosidase and β-galactosidase of emulsin type. The present experiment indicated that this β-glucoside was able to inhibit also the β-xylosidase activity of emulsin type. In a similar manner the activities of β-glucosidase and β-xylosidase of emulsin type are inhibited by phenyl β-xyloside. However the result that this β-xyloside which belongs to a strong inhibitior of *p*-nitrophenyl β-xyloside hydrolysis by Taka-enzyme inhibited though to a less extent also the Taka β-glucosidase, remains at present unexplained.

Similar to the previous result that the mode of inhibition by the lactones of hexonic acids did not differ from that by phenyl β-hexosides, it was observed that xylonolactone acts in the same way as phenyl β-xyloside. However, its inhibitory activity is not stronger than that of phenyl β-xyloside, contrary to the results previously obtained that the lactones of hexonic acids were the most active inhibitors. Regarding also no inhibitory effect of xylonate to Taka β-xylosidase activity, it may be said that the affinities of xylonate and xylonolactone toward the enzymes are generally weaker than those of hexonates and their lactones.

From the results mentioned above and the natural occurrence of β-glucosidase and β-galactosidase free from β-xylosidase it should be definitely said that β-xylosidase is a specific enzyme and two types are to be differentiated in it. β-Glucosidase and β-glucuronidase are already known to be enzymes different from each other (*11*, *12*), hence no inhibitory activity of glucuronate, glucosaccharate, and mucate upon the three Taka β-heterosidases may be well understood. Oxdiation of terminal alcohol group on position 6 of glucose residue of glucoside to carboxyl or replacement of that group by hydrogen atom causes the original β-glucoside to become susceptable to the β-glucuronidase and β-xylosidase actions, respectively.

It was our unexpected result that glucosaccharolactone, contrary to xylonolactone, had no effect upon the three heterosidases of emulsin type in spite of the presence of γ-lactone ring in the molecule as in gluconolactone and galactonolactone, though our preparation of glucosaccharolactone applied for the test may have been a mixture of 1, 4 and 3, 6-lactones (*13*). At present it should be said that oxidation of terminal alcohol group of hexonolactone to carboxy would lower the affinity toward β-heterosidases of that lactone ring.

SUMMARY

p-Nitrophenyl β-xyloside is specifically hydrolysed by β-xylosidase. For this enzyme there are two different, namely Taka and emulsin, types. They are differentiated by the mode of substrate-enzyme complex formation. The enzyme of Taka type unites at first with xylose residue of β-xyloside, while in the case of emulsin type the first union with the substrate takes place at the β-heteroside linkage. The hydrolysis by the Taka β-xylosidase is inhibited by xylose, phenyl β-xyloside and xylonolactone, but not at all by other sugars and their corresponding derivatives, while the activity of emulsin β-xylosidase is inhibited by phenyl β-xyloside as well as by phenyl β-glucoside. Xylonolactone inhibits not only β-xylosidase of emulsin type also β-glucosidase and β-galactosidase of the same type. However, monolactones of saccharic acids lack an inhibitory effect upon all β-heterosidases of both types.

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INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

V. SPECTROSCOPIC AND ELECTROPHORETIC INVESTIGATIONS ON THE REACTION OF METHEMOGLOBIN WITH SODIUM DODECYLSULFATE

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(Received for publication, November 8, 1955)

As reported by Tsushima (*1*), the absorption specturum of methemoglobin solution changes to that of hemichrome by addition of sodium salicylate or sodium benzoate in their definite concentrations.

It has been assumed by Tsushima that this absorption figure of hemichrome may be ascribed to the coordination structure similar to that of pyridine hemichrome, namely, to the bonding of globin N from both sides of its heme Fe. The bonding of another globin N to heme Fe, which is otherwise incapable of bonding in the natural molecule, may have been resulted by the opening out of bonding groups through some structural modification of methemoglobin molecule caused by the addition of perturbators such as salicylate or benzoat.

To examine the interrelation between the absorption change of methemoglobn and the grade of structural modification of its globin moiety induced by the perturbators, the authors intended to investigate the absorption changes of methemoglobin caused by addition of sodium dodecyl sulfate (SDS), which has often been used as a protein denaturant, and to study the interaction of globin molecule with SDS by means of electrophoretic method.

That the absorption figure of methemoglobin solution changes by the various synthetic detergents as well as by salicylate and benzoate, had already been reported by Anson (*2*). And the interaction between the serum albumin and SDS has been precisely studied by Putnam and Neurath (*3*). However, the quantitative relationship between the structural modification of globin part and the absorption change of methemoglobin has not yet been studied intentionally.

EXPERIMENTS

Materials and Method

Methemoglobin solution for our experimental purpose was prepared as follows. Crystalline equine oxyhemoglobin was prepared following the method of Heidelberger (*4*), improved in some points by Suzuki *et al* (*5*). This was dissolved in distilled water. Sodium nitrite, twice as much as heme concentration, was then added to this oxyhemoglobin solution in order to convert hemoglobin oxidatively to methemoglobin. The excessive nitrite was removed by dialysis.

Crystalline sodium dodecyl sulfate was dissolved in distilled water as a stock solution, pH of the solution being adjusted to 7.8 with diluted hydrocholoric acid solution. The stock solutions of methemoglobin and SDS were diluted with $M/15$ phosphate buffer (pH 7.8) at the spectroscopical observations.

In the electrophoretic studies, the stock solutions of methemoglobin was preliminary dialysed against $M/20$ phosphate buffer (pH 7.8) overnight. SDS solution was diluted with $M/20$ phosphate buffer (pH 7.8) every time at use.

For spectroscopic measurements, a photoelectric spectrophotometer Model FPB-V of Hitachi Company were used, and for electrophoretic studies, a Tiselius apparatus of Hitachi Company were used. The electrophoretic measurements of methemoglobin-SDS mixtures were run for the same time length (60 minutes) at the same field strength of 0.4 mA./mm^2. The temperature was 11°.

RESULTS

Spectroscopical Observations

The absorption figure of methemoglobin at pH 7.8 changes to that of hemichrome by addition of $5\times10^{-3}\,M$ of SDS as shown in Fig. 1, I. This absorption figure is similar to that of hemichromes obtained by the addition of perturbators such as salicylate, benzoate or KI. This absorption spectrum may thus be ascribed to a coordination structure, as in the case of pyridine hemichrome.

The reactions between methemoglobin and SDS were then more precisely and quantitatively studied. A series of different absorption figures were obtained by SDS according to its varying concentrations in the range below $5\times10^{-3}\,M$, as shown in Fig. 1. Two isosbestic points at 515 and 595 mμ were clearly demonstrated in the figure, so that the system may well be inferred to be composed of two components.

The percentage of hemichrome formed was calculated following the procedure described in our previous paper. The percentages of hemichrome formation at every SDS concentration were plotted against log concentrations of SDS as shown in Fig. 2. The concentration of

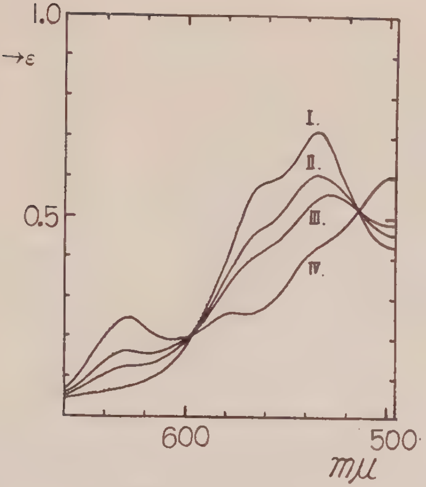

Fig. 1. The absorption figures obtained by addition of varying SDS concentrations.

Concentration of methemoglobin (heme concentration): 6.25×10^{-5} M.

Concentrations of SDS: I, 5×10^{-3} M; II, 2×10^{-3} M; III, 1.5×10^{-3} M; IV, 0.0 M. pH: 7.8.

SDS required for the hemichrome formation was found to be much lower than those of salicylate and benzoate.

Now, when the same experiment was carried out with different concentrations of methemoglobin, the relationship between the percentage of hemichrome formation and the concentration of SDS was found to be all the same, but the curve shifted along the horizontal axis according to the difference of methemoglobin concentrations. This result means that the law of mass action is inapplicable to this reaction. If the mass law is applicable to this reaction, such a shift of the curve may never be found, for the concentration of SDS was held excessively higher than that of methemoglobin in this experiment.

Fig. 3 shows a relationship between the percentage of hemichrome formation and the ratio of the concentrations of SDS and methemoglobin. It is obvious from the figure that the hemichrome formation is dependent on the concentration ratio of SDS to methemoglobin.

Electrophoretic Studies

It was found, as described above, that the hemichrome formation

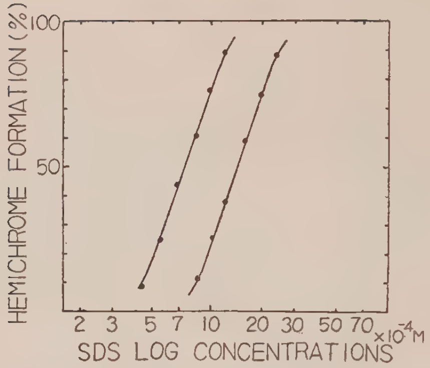

FIG. 2. Plots of the percentage of hemichrome formation *vs.* log. concentration of SDS.

Concentrations of methemoglobin (heme concentration) I, 3.77 × 10^{-5} *M*; II, 7.54 × 10^{-5} *M*. pH: 7.8.

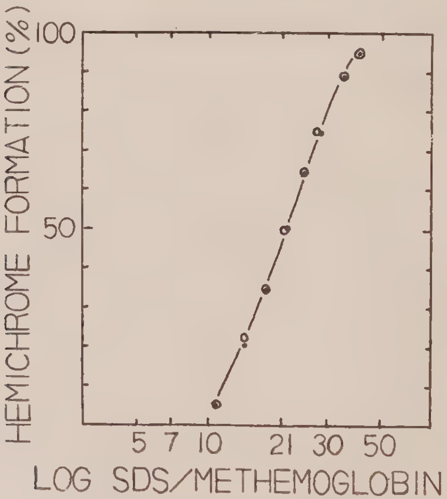

FIG. 3. Plots of the percentage of hemichrome formation *vs.* log. SDS/methemoglobin.

Concentrations of methemoglobin (heme concentration)

–●–●– 7.54 × 10^{-5} *M*

–○–○– 3.77 × 10^{-5} *M*

is variable according to the ratio of SDS and methemoglobin concentrations. Thus methemoglobin-SDS mixtures of constant methemoglobin concentration and varying SDS concentration were prepared by mixing the stock solutions and diluting with buffer to a definite volume. The absorption changes of methemoglobin by addition of SDS was in completion in a few minute and the resulted absorption figure remained unchanged during few hours. Consequently, to insure the complete interaction between methemogobin and SDS, the mixture must necessarily be allowed to stand at least over few minutes at room temperature before electrophoretic measurements.

A typical figure of the electrophoretic diagrams, reproduced from the original plates, are given in Fig. 4. The arrows in the diagrams indicate the direction of migration. Since all of the photographs were taken after electrophoresis for the same length of time (1 hour) and at the same field strength, the mobilities of the respective components may be compared directly by the distance of migration from the original point. As shown in Fig. 4, there appeared several boundaries corresponding, in the order of their increasing mobility, to the methemoglobin (A), a first complex (I), a second complex (II), and a trace of another substance (D) seemingly that of the detergent. By addition of SDS to methemoglobin, the boundary (I) appeared firstly. This boundary (I) disappeared by increasing addition of SDS, and then a new second boundary (II) appeared. Finally, only this second boundary remained even by an excessive addition of SDS.

From the relationship between the electrophoretic behavior of mixtures of methemoglobin-SDS and the respective ratio of SDS and methemoglobin concentrations as shown in Fig. 4, it was known that the boundary of methemoglobin (A) almost disappears in the ratio of SDS to methemoglobin of 40:1.

This ratio corresponds to that by which the absorption of methemoglobin changes completely to that of hemichrome as described in section 1. With increasing ratio of SDS to methemoglobin above 40, there appeared secondary change in the electrophoretic behavior. In this concentration range, however, no further absorption change were observable at least in the visible region.

DISCUSSION

From the electrophoretic studies presented here, it is suggested that the electrostatic binding of methemoglobin with SDS may be a stoichiometric one as an " all or none " phenomenon at least in the first stage,

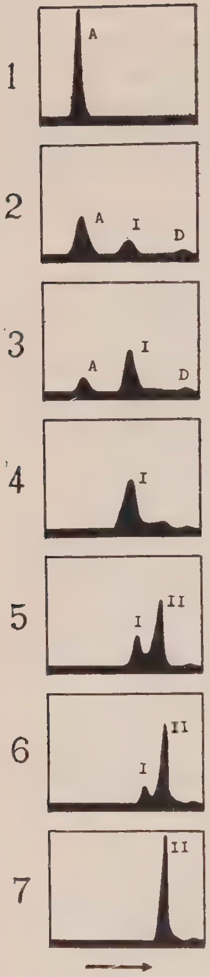

FIG. 4. Electrophoretic analysis of methemoglobin-SDS mixtures at 11°.

SDS to methemoglobin ratio:

1. Methemoglobin alone.
2. 13.3
3. 20.0
4. 40.0
5. 53.5
6. 70.0
7. 100.0

pH: 7.8 (1/20 *M* phosphate buffer)

All experiments were run for the same time (60 minutes) at the same field strength (0.4 mA./mm.^{2}). The components A, I, II, D indicate respectively methemoglobin, first complex, second complex, and detergent.

The arrows denote the direction of migration. (Descending boundary).

namely corresponding to that of the disappearance of boundary A. Since, the absorption changes of methemoglobin to hemichrome were observable in the first stage, the methemoglobin reacting with SDS as an " all or none " phenomenon, it may be assumed that the structural modification of globin molecule allowing a coordinative linking of another globin N to heme Fe, may have been brought about by stoichiometric binding of SDS to methemoglobin. And from the fact that the absorption changes of methemoglobin into the complex I in electrophoresis, it may be concluded that the absorption changes are also to be a nature of " all or none " phenomenon.

Appearance of complex II as a result of further increase in the ratio of SDS to methemoglobin, indicates a multiple combination of SDS molecules to the globin molecule. In a reaction between serum albumin and SDS, Putnam and Neurath (*3*) have assumed that the reaction stage of the complex II formation obserbable electrophoretically, are the binding reaction of SDS with cationic group as a substitute for a hydrogen bond between peptide chains. And they assumed that the structural modification in the molecule of complex II, may be more intensive than in that of complex I. By analogy with their assumption, the stuructural modification, possivly the unfolding of peptide chains of complex II of methemoglobin, seems to be of more progressed one than that of the complex I. In a stage before occurence of more intensive configurational changes, there seems to occur a linking of another globin N to heme Fe. This assumption seems to be of some importance for the support of our views that the reversible absorption changes occuring by addition of salicylate and benzoate may be accounted for a structural modification of globin part in a more slightly grade.

It is reported by Kikuchi (*6*) that the oxidative activity of oxyhemoglobin increases by addition of salicylate and benzoate in much lower concentration than that required for the actual absorption change, and that the perturbation of hemoglobin molecule seems to proceed stepwise following the increase of salicylate and benzoate concentration.

Also from our experimental results, it may be postulated that the structural modification of globin molecule seems to proceed stepwise. No absorption change, at least in the visible range, could be observed at the reaction step of complex II formation from complex I. At the reaction step of complex I formation, however, there appeared a definite absorption change.

It seems to be possible, thus, to characterize the structural modification of globin moiety of heme proteins from their absorption change in the visible region as one of the criteria.

SUMMARY

1. The absorption figure of methemoglobin changes to that of hemichrome by addition of sodium dodecyl sulfate. The hemichrome formation is dependent to the concentration ratio of SDS to methemoglobin.

2. The electrophoretic diagrams of methemoglobin SDS mixtures show three different boundaries corresponding, in the order of their increasing mobility, to methemoglobin, a first complex and a second complex in accord with the increasing concentration ratio of methemoglobin to SDS.

3. The progress in the hemichrome formation is dependent on the formation of the complex I.

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METABOLISM OF ANTHRAQUINONE

I. ISOLATION OF 2-HYDROXYANTHRAQUINONE FROM THE URINE OF RATS

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(Received for publication, November 14, 1955)

The metabolism of anthracene has been studied by *Boyland et al.* (*1*, *2*) and 1, 2-dihydroxy-1,2-dihydroanthracene and its 1-glucuronide were isolated. However, there has been no report on the metabolism of anthraquinone. In the present work the isolation of its metabolite was studied in rats and from ether extract of the urine 2-hydroxyanthraquinone was obtained.

EXPERIMENTAL AND RESULS

Dosing of Animals—The animals used were rats weighing from 150 to 200 g. They were housed in metabolism cages designed to permit the collection of urine separate from faeces. The animals were fed on a diet consisting of starch, casein, oil and minerals with vitamin supplements.

Each animals received 100 mg. of anthraquinone mixed with the diet.

Preparation of Urine Extracts—Urine was collected and stored in a refrigerator. About 30 ml. of the urine was filtered, continuously extracted with peroxide-free ether for 24 hours, and the ether extract was evaporated to dryness. A brownish yellow residue was obtained.

Isolation of Yellow Crystals—The residue was dissolved in a small volume of ethanol and a small portion of distilled water was added until it became slightly turbid. The precipitate was centrifuged and discarded. The supernatant was added with a small volume of distilled water, left overnight, and a precipitated product was collected by centrifuging.

Properties of the Product—It was almost insoluble in cold water and melted with decomposition at 306° (corr.). When this substance was mixed with 2-hydroxyanthraquinone the melting point was not despressed. It sublimed at about 200° to yellow plates. It is very soluble in ethanol and ether to form a yellow solution. It is soluble in sodium hydroxide solution, barium hydroxide solution, and concentrated sulfuric acid to form a reddish-yellow solution. The absorbtion was measured with the sublimed crystals in 50 per cent of ethanol, 0.1 N NaOH, or conc. H_2SO_4 as shown in Fig. 1. The curves were identical with those of the authentic sample of

2-hydroxyanthraquinone prepared by Dr. Shibata. They showed good agreement with those reported by Meyer *et al.* (*3*) in wave length above 250 mμ in alkali and conc. H_2SO_4, but showed a little difference from that reported by Moir (*4*).

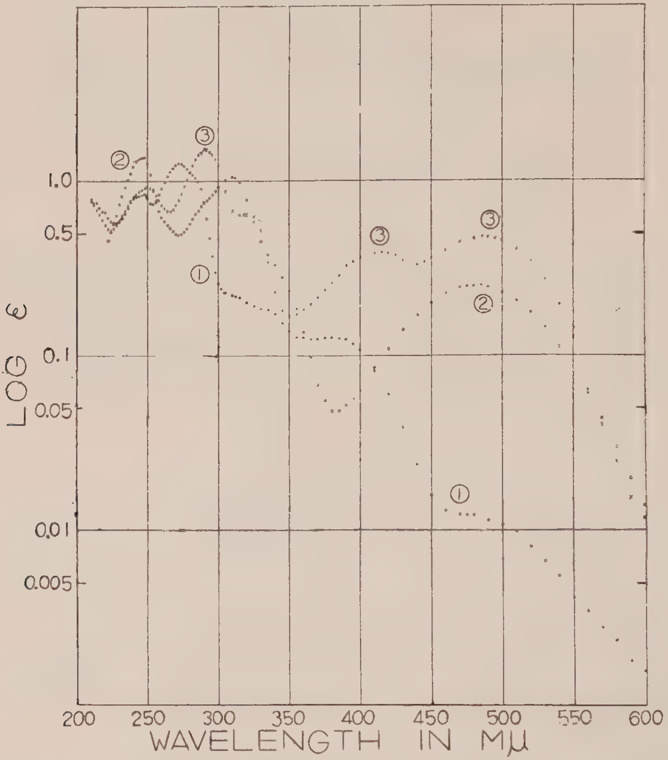

FIG. 1. Ultraviolot spectra of the yellow substance (2-hydroxy-anthraquinone).

① in 50 cer cent ethanol solution.
② in about 0.1 *N*-NaOH-ethanol solution.
③ in conc. H_2SO_4.

Methylation of the Compound—These yellow crystals were dissolved in methanol, hydrogen chloride was passed through the solution and the solution was heated at 60° for 30 minutes. It yielded brilliant yellow crystals, which sublimed above 100° and melted at 195° (corr.) with browning. These properties correspond to 2-methy-oxyanthraquinone. Its absorbtion curves in ethanol and 0.1 *N* NaOH were shown in Fig. 2.

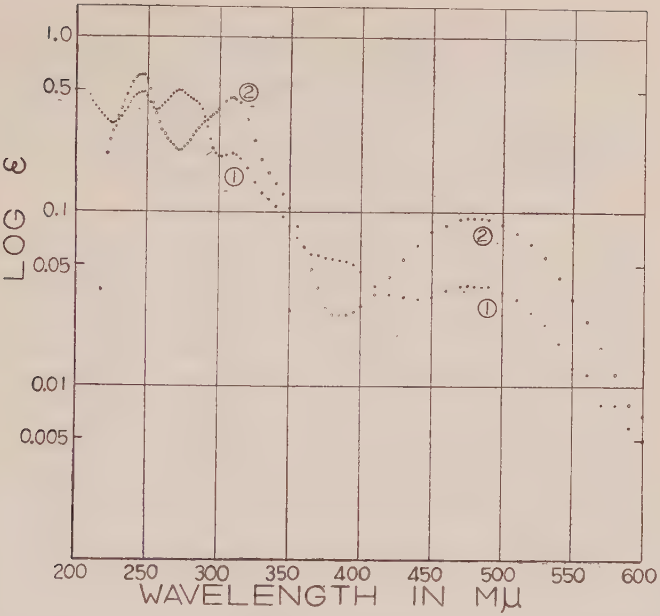

FIG. 2. Ultravilet spectra of the methylated yellow substance. (2-methoxyanthraquinone).
① in 50 per cent ethanol solution.
② in about 0.1 *N*-NaOH-ethanol solution.

Paperchromatography—Ascending chromatography on a Whatman No. 1 paper was carried out with the solvent of butanol, water, acetic acid (4:1:1). The yellow substance ascended to the top. With methanol-saturated petroleum ether described by Shibata (*5*), it showed the Rf value of about 0.3, identical with the anthentic sample of 2-hydroxyanthraquinone. There was a very faint yellow spot at the top of the solvent front with methanol-saturated pertoleum ether when the ether extract of the urine was used, and this substance changed to reddish yellow when treated with alkali. These properties are common to 1-hydroxyanthraquinone. This shows that a trace of 1-hydroxyanthraquinone is excreted in the urine of rat dosed with anthraquinone.

DISCUSSION

The yellow substance obtained in ether extract of the urine showed a good agreement with 2-hydroxyanthraquinone, but its quantity was only several per cent of the dosed anthraquinone.

The effort to obtain 1-hydroxyanthraquinone from the ether extract of the urine by sublimation at 80° was not successful but a trace of the substance seemed to be present on the paper chromatogram. It is interesting that the 2-position of anthraquinone is oxidised. Several metabolites other than 2-hydroxyanthraquinone can be detected by paper chromatography of the urine by fluorecence or S^{35}-sulfate conjugation method, but further studies are needed.

SUMMARY

1. A study was made on the metablism of anthraquinone by oral dosing.

2. Yellow crystals were isolated from ether extract of the urine either by recrystallisation with ethanol and water or sublimatisation at about 200°.

3. The properties of the isolated substance was examined by its melting point, solubility in organic solvents and alkalis, and the absorbtion measurements, in 50 per cent ethanol, 0.1 *N* sodium hydroxide solution, and concentrated sulfuric acid. These properties showed a good agreement with those of 2-hydroxyanthraquinone.

The authors wish to express their gratitude to Prof. Shibata of the Tokyo University for supplying them with hydroxy derivatives of anthraquinone and Dr. Yoshikawa for his help in these experiments.

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CONJUGATION OF S^{35}-SULFATE WITH PHENOLIC COMPOUNDS

II. ON THE SULFATE CONJUCATE OF 1,2-DIHYDROXYNAPHTHALENE IN THE URINE OF RATS DOSED WITH NAPHTHALENE

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The metablism of naphthalene has been studied by Young *et al.* (*1–4*) and by Boyland *et al.* (*5, 6*) and various metabolites have been isolated from the urine. In the present work the sulfate conjugates of metabolites of naphthalene in the urine of rats were studies by paper chromatography and new metabolite was found. From the chemical properties, the new substance seemed to be a sulfate conjugate of 1,2-dihydroxynaphthalene. Its metabolic relation to 1,2-dihydronaphthalene-1,2-diol was examined *in vitro* experiments with rat liver.

EXPERIMENTAL

Dosing of Animals—The animals used were rats weighing from 150 to 200 g. They were housed in metabolism cages designed to permit the collection of urine separate from the faces. The animals were fed on a diet consisting of starch, casein, oil and minerals with vitamin supplements. Each animal received 100 mg. of 1-naphthol, 2-naphtol or naphthalene mixed with the diet. For the study of sulfate conjugates, S^{35}-sulfate was injected subcutaneously and the urine was collected for about 5 hours after the injection.

Chromatographic Procedures—Ascending chromatography on Whatman No. 1 paper was used, and the solvents employed was a mixture of *n*-butanol: acetic acid: water (4:1:1) or the solvent system employed by Young *et al.* (*4*). The chromatograms were developed for about 16 hours.

Preparation of Urine Extracts—Urine was collected, stored in a refrigerator and filtered. The urine or its *n*-butanol extract was used for chromatography.

Detection of Compounds on Paper Chromatograms—For the detection of naphtholic substances, the papers were sprayed lightly with a solution of diazotized sulfanilic acid followed by 1*N*-NaOH. To detect the conjugates of naphtholic substances, the conjugates were hydrolysed on chromatograms by suspending the papers in fumes of HCl at room temperature for 1 hour. The papers were then exposed to a current of air and naphthols liberated by acid hydrolysis were detected as already described. S^{35}-sulfate conjugates were detected by radioautography using Fuji No-Screen Films.

Experiments with Liver Slices—The experiments were conducted under conditions already described by the authours (*7*) in microscale. Inorganic S^{35}-sulfate was precipitated by excess of barium chloride after the addition of carrier sulfate, and the excess barium was precipitated by excess of non-isotopic sulfate. The supernatant was developed as already described.

Experiments with the Supernatant of Liver Homogenate—The liver was homogenated with equal amount of the mixture (8:2 in volume) of 0.154 *M* potassium chloride and magnesium chloride, and the supernatant was obtained by centrifuging at about $10,000 \times g$. and fortified with potassium salt of ATP. The experimental condition was as described under Fig. 3.

RESULTS

Experiments with the Urine of Animals Doesd with 1-Naphthol or 2-Naphthol.

By radioautogram (Fig. 2) the urine of rats dosed with 1-naphthol showed a large spot of S^{35}-sulfate at the middle of the paper and several small spots of smaller Rf. The large spot did not show immediate diazo reaction but after one or two days red color appeared.

After incubation in fumes of HCl, it showed instant diazo reaction of 1-naphthol. The section of the paper strip which carried the large spot was cut out, immersed for 10 minutes in warm water, and hydrolysed for 10 minutes in *N*-HCl at 100°. The presence of 1-naphthol was detected by chromatography and color reaction.

It may be concluded that the large spot of 1-naphthol-dosed urine detected by radioautogram of S^{35}-sulfate was that of 1-naphthyl sulfate as already described by Corner and Young (*4*). The same conclusion was reached for the large spot from the urine of an animal dosed with 2-naphthol.

Experiments with the Urine of Animals Dosed with Naphthalene

Administration of naphthalene also resulted a large spot with almost the same Rf value as in the case of 1- and 2-naphthol by radioautogram (Fig. 2). This spot, however, showed immediate red diazo raection, the color changing to purple with time. This shows that this sub-

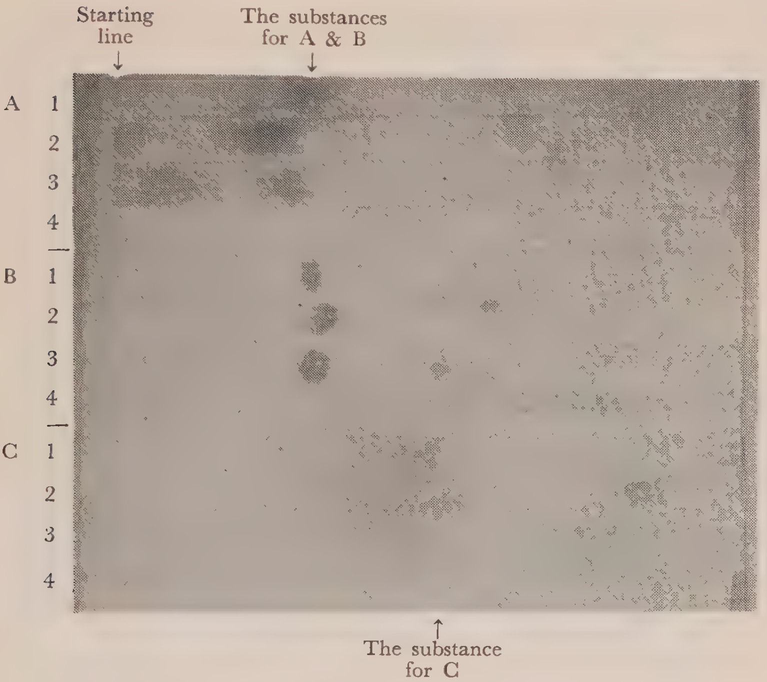

FIG. 1. Diazo reaction of paper chromatograms of the urine of animal dosed with naphthols and naphathalene (after 4 weeks).
Solvents A: $3N\text{-}NH_4OH—3N(NH_4)_2CO_3$—$n$-butanol (3:3:4)
B: $3N\text{-}NH_4OH—3N(NH_4)_2CO_3$—iso-Butanol (3:3:4)
C: CH_3COOH, H_2O, n-butanol (1:1:4)
Dosed substance
1: 1-Naphthol
2: 2-Naphthol
3: Naphthalene
4: None

stance has a free hydroxylgroup.

On hydrolysis in the fumes of HCl for 1 hour, the spot turned to faint brown and no color reaction was observed by diazo reagents. After elution and hydrolysis of the spot by $1N$-HCl, it proved to contain a small quantity of 1-naphthol by chromatography and diazo reaction. This indicates that the main part of large spot is as being different from 1-naphthyl or 2-naphthyl sulfate.

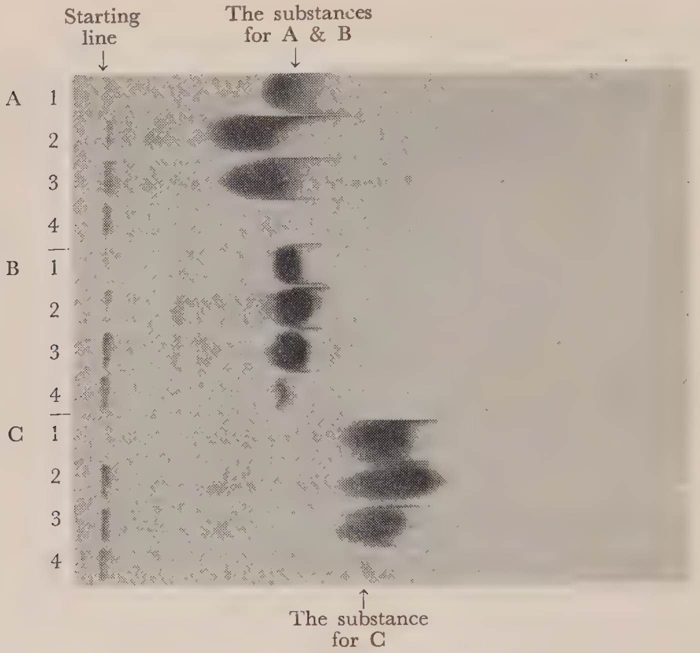

Fig. 2. Radioautograms of the paper chramatograms of Fig. 1.

Hydrolysis of the eluate in 1*N*-HCl changed the color to pale yellow and the substance was easily extracted with ether. When the eluate was made alkaline with sodium hydroxide solution, it gave a brown precipitate and became difficult to extract with ether. It turned to pale yellow by addition of hydrosulfite in alkaline solution.

After hydrolysing the spot in 1*N* HCl, made alkaline with NaOH solution and extracted with peroxide-free ether to remove the the small amount of 1-naphthol, the aqueous layer was acidified with HCl and extracted with peroxide-free ether. The ether layer was spotted on a paper and dried. This spot changed to deep green with concentrated sulfuric acid. These properties suggest that the hydrolysate mainly consists of 1,2-naphthoquinone. This substance was dissolved in alkali with hydroxylamine hydrochloride, heated for a while, spotted on filter paper, and chromatographed with *n*-butanol-saturated water. This showed the same spot as that of 1,2-naphthoquinone.

In Vitro Studies with 1,2-Dihydroxynaphtalene.

1,2-Dihydroxynaphthalene (m.p. 60° from water) was prepared from 1,2-naphthoquinone and incubated with liver slices or with supernatant of a liver homogenate. As seen in Fig. 3, the S^{35}-sulfate conjugate of 1,2-dihydroxynaphthalene appeared as a marked spot with almost the same Rf value as the large spot of 1-naphthol when incubated with supernatant of homogenate. In the case of slices the spot was very faint.

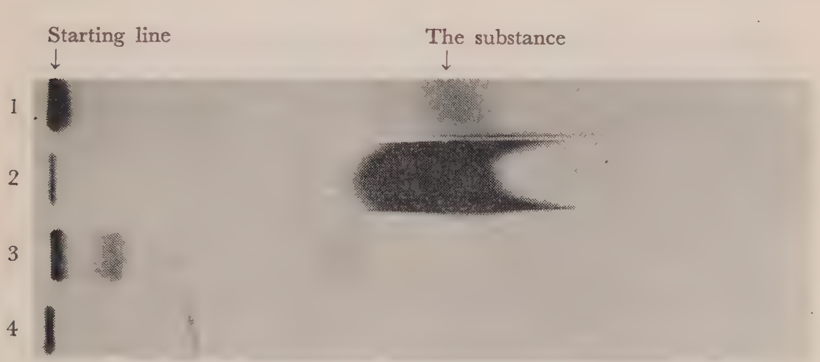

FIG. 3. Radioautograms of the sulfate conjugate derived from 1, 2-dihydroxynaphthalene, 1-naphthol or naphthalene in the presence of in the presence of rat liver preparations.

1: 1, 2-Dihydroxynaphthalene, with supernatant.
2: 1-Naphthol, with supernatant.
3: Naphthalene, with supernatant.
4: 1, 2-Dihydroxynaphthalene, with slices

Incubation, with supernatant:

Supernatant (50 per cent) 0.1 ml.+KATP 1.5 mg.+naphthol or naphthalene 20 μg.+S^{35}-sulfate, shaken in air phase at 37.5° for 1 hour.

Incubation, with slices:

Slices 0.1 g.+Ringer-phosphate-succinate (without SO_4) 0.1 ml. +naphthol or naphthalene 20 μg.+S^{35}-sulfate,

Shaken in oxygen at 37.5° for 1 hour.

After deproteinization with trichloracetic acid, inorganic S^{35}-sulfate was removed with $BaCl_2$. Solvent for chromatography: NH_4OH $(NH_4)_2CO_3$—isobutanol.

In Vitro Studies with Naphthalene

Naphthalene was dissolved in Methyl-Cellosolve and incubated with S^{35}-sulfate. As shown in Figs. 3 and 4, a large spot of the sulfate conjugate with Rf close to that of 1-naphthol was seen in the case of liver slices but very faint in the case of the supernatant.

In Vitro Studies with 1,2-*Dihydronaphthalene*-1,2-*diol*

1,2-Dihydronaphthalene-1,2-diol was isolated from rat urine according to the method of Corner *et al.* (*3*). In this case, the results were just the same as with 1,2-dihydroxynaphthalene. (Fig. 4).

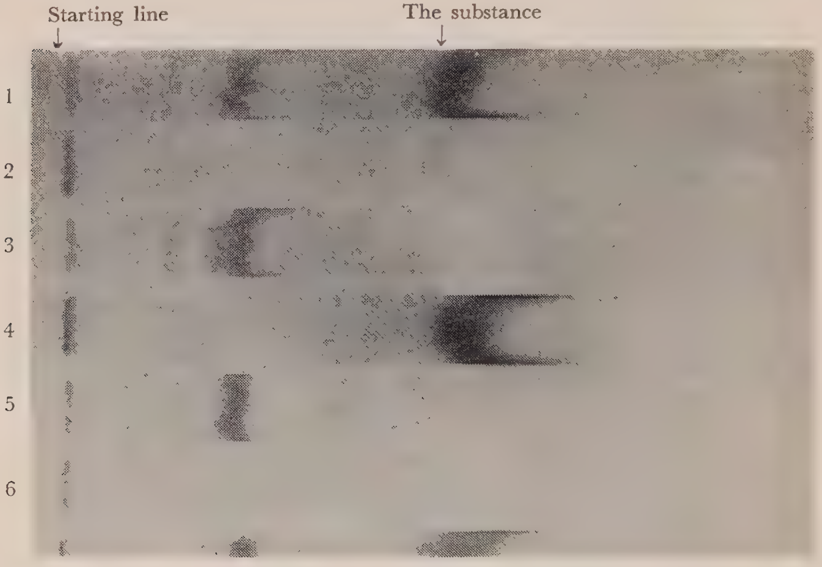

Fig. 4. Radioautograms of the sulfate conjugate derived from 1, 2-dihydronaphthalene-1, 2-diol or naphthalene in the presence of rat liver preparation.

1: 1, 2-Dihydronaphthalene-1, 2-diol, with supernatant.
2: 1, 2-Dihydronaphthalene-1, 2-diol, with slices.
3- Naphthalene, with supernatant.
4: Naphthalene, with slices.
5: Control, with supernatant.
6: Control, with slices,

Conditions were the same as in Fig. 3. A nonspecific radioactive spot spot is seen in 5 by the supernantant, but disapperared in 6 by slices.

DISCUSSION

Main part of the sulfate conjugate, which was found in the urine of rats dosed with naphthalene, was proved to be different from 1-naphthyl- or 2-naphtyl sulfate, because it showed an immediate diazo reaction before hydrolysis and no color reaction with diazo reagents after hydrolysis. Immediate diazo reaction shows that it has a free hydroxyl radical. After hydrolysis, it showed the properties of 1,2-naphthoquinone. The S^{35}-sulfate conjugate of 1,2-dihydroxynaphthalene showed the same Rf value as that of the substance from urine.

From these results, it may be concluded that the sulfate conjugate found in the urine of naphthalene-dosed animals is a sulfate conjugate of 1,2-dihydroxynaphthalene.

1,2-Dihydroxynaphthalene seems to be derived from 1,2-dihydronaphthalene-1,2-diol in the supernatant of liver homogenate, but in the case of liver slices, those two substances are very liable to undergo further metabolism. Naphthalene seems very difficult to be metabolised to 1,2-dihydronaphthalene-1,2-diol in the supernatant, because the spot of the sulfate conjugate did not appear. In liver slices, however, 1.2-dihydronaphthalene-1,2-diol may be continuously produced and may become the source for the sulfate conjugation of 1,2-dihydroxynaphthalene. Boyland (*6*) showed that the recovery of the dosed 1,2-dihydronaphthalene-1,2-diol from the urine was very poor, and this can be explained by the results of above in vitro experiments.

SUMMARY

A study has been made of the sulfate conjugate of the metabolite in the urine of rats dosed with naphthalene, by the aid of S^{35}-sulfate.

The excretion of a sulfate conjugate, which showed immediate diazo reaction before hydrolysis and no color reaction with diazo reagents after hydrolysis was confirmed by paper chromatography.

This substance gave a spot corresponding to 1,2-naphthoquinone by acid hydrolysis.

When 1,2-dihydroxynaphthalene and 1,2-dihydronaphthalene-1,2-diol was incubated with S^{35}-sulfate in the supernatant of liver homogenate, a spot corresponding to the above sulfate conjugate in the urine was detected. The spot was faint in the experiment with liver slices.

Naphthalene gave a spot with the same Rf value when incubated with liver slices, but with the supernatant, the spot was very faint.

Considering these results, the sulfate conjugate of naphthalene metabolite may be 1,2-dihydroxynaphthyl sulfate and its metabolic

position in relations to naphthalene and 1,2-dihydronaphthalene-1,2-diol was discussed.

An account of the work described in this paper was presented at the 77th meeting of the Japanese Biochemical Society is Kanto District at Tokyo University on June 7, 1955. After the meeting, a report describing the sulfate conjugate of 1,2-dihydroxynaphthalene from naphthalene and 1,2-dihydronaphthalene-1,2-diol was presented by Corner and Young (*8*) in the Biochemical Journal, September 1955.

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DEGRADATION OF STARCH IN THE ENDOSPERMS OF RICE SEEDS DURING GERMINATION[1]

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Starch in general comprises two fractions, amylose and amylopectin in a varying ratio among various plant sources. Recent investigations showed that the ratio is not always constant even for the same plant source varying with its development stages. Bice *et al.* (*1*) and Wolf *et al.* (*2*) reported that the starch in the immature seeds of wheat and corn have the lower contents of amylose than in the mature, a fact which confirmed later on various plants by some investigators (*3, 4, 5*). Potato starch during the storage was analyzed by Schwimmer *et al.* (*6*), but no distinct discrepancy was obtained. Although it is known that the starch in cereal seeds is degraded so rapidly during germination, no study has been made on their characteristics.

This paper deals with some characteristics of starch in the endosperms of rice seeds, *Oryza sativa*, during germination. The result showed amylose-amylopectin ratio of the starch gradually increased as the degradation proceeded.

MATERIALS AND METHODS

Rice Seeds—Rice seeds[2], Yamada-Nishiki, harvested in Autumn of 1954 were used. Glutinous rice seeds, hybrid of Asahi and Takao, harvested in Autumn of 1954 were kindly supplied by Dr. Suzuta to whom the authors express their gratitude.

Germination—Dehulled seeds were macerated in distilled water for one hour at 30° and planted on an agar bed (0.5 per cent agar, no nutrient was supplied) and kept in the dark at 30±1°. The embryonic plants of various ages were separated into three parts: shoot[3], root, and endosperm. As zero time control dehulled seeds after ordinary maceration were employed.

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[1] A part of this article was presented at the Annual Meeting of the Agricultural Chemical Society of Japan held in Tokyo on March 31, 1955.

[2] Unless otherwise stated, rice means non-glutinous species in this report.

[3] Shoot included plumule, coleoptile, and scutellum.

Determination of Dry Weight—An appropriate pieces of excised organs were dried at 100±2° until constant weights were obtained. The dry matters were cooled in a dessicator over calcium chloride before weighing.

Preparation of Starch Samples—Usual methods for preparing the starch samples from the intact starch granules in plant source (*7*) might be not suitable for the damaged granules or free starch molecules which might occur in the course of degradation. McCready *et al.* (*8*) reported a new method for analyzing starch in vegetables, which comprised the dissolution of materials in perchloric acid after repeated extraction with hot eighty per cent ethyl alcohol. Starch samples of various ages were prepared from 50 to 100 excised organs according to this method (*8*).

Analytical Procedures—Starch contents and blue values[4] of the above samples were determined by the method of McCready *et al* (*8*). In some experiments alcohol-residues were defatted by extraction with four changes of seventy per cent methyl alcohol each for three hours. Absorption curves of starch-iodine complexes were taken of the solution containing 5 mg. of starch, 10 mg. of iodine, and 100 mg. of potassium iodide in 200 ml., using the photoelectric spectrophotometer (Hitachi Seisakusho, 0.1 mm. of slit width and 10 mm. quartz cell). To determine the iodine affinity, the potentiometric titration method (*9, 10*) were applied to the starch samples obtained from the alcohol-residue after repeated filtration through fine nylon cloth.

RESULTS

Dry Weight—Changes in dry weight of each organs were estimated (Fig. 1). Under the conditions used (agar-culture at 30° in the dark), growth of the sprout ceased on about the tenth day after planting.

Starch Content—Changes in the starch content of each organ during germination were determined according to the anthrone method (*8*), and showed in Fig. 2. It is seen that the endosperms decrease in starch content roughly in parallel to the loss in dry weight in the earlier germination stage. Starch contents of the shoot and root tend to increased gradually, but were far less than that of the endosperm. Their amounts was not sufficient to investigate their chemical nature.

Blue Value—Table I (a) shows the typical results of the blue values of starch samples from the endosperms during germination; they exhibited higher blue values as the germination proceeded. The defatted materials showed invariably higher values than the corresponding

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[4] Blue value was tentatively defined as the optical density of the solution containing 5 mg. of starch, 10 mg. of iodine, and 100 mg. of potassium iodide in 500 ml., measured by the photoelectric colorimeter (AKA-type of Kotaki Seisakusho, using the S-66 filter and 20 mm. glass cell).

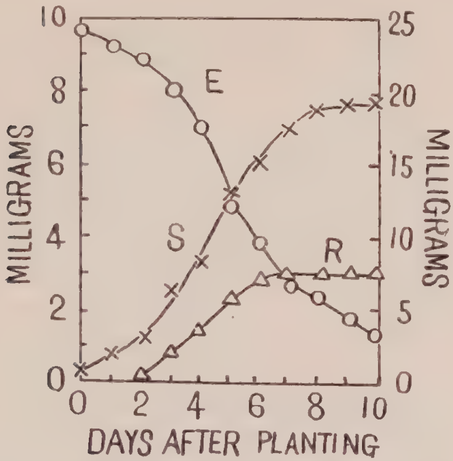

FIG. 1. Changes in the dry weight of embryonic organs of rice during germination.

E: endosperm.
S: shoot.
R: root.

Scales on the right-hand ordinate, for E; the left-hand, for S and R. Values per one individual are plotted.

non-defatted specimens. It would be concluded that the relative content of amylose in starch in the endosperm increases during germination. Such a finding has not been reported with regard to any germinating seeds of higher plants.

The increase in the blue value was also discernible in starch of germinating glutinous rice seeds, while their increasing values were fairly small as compared with that of non-glutinous rice seeds. (Table I (b)).

Absorption Curve—Absorption curve of starch-iodine complex using the starch sample taken on the 6th-day after planting was shown with that of the non-germinating seeds in Fig. 3. No distinct shift in maximum absorption is seen in the two curves. Here again the higher optical density of the starch in the endosperm of germinating seeds was attained.

*Iodine Affinity*5)—To confirm this findings, the potentiometric ti-

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5) We wish to thank Mr. F. Hattori for his assistance in the measurement of iodine affinity.

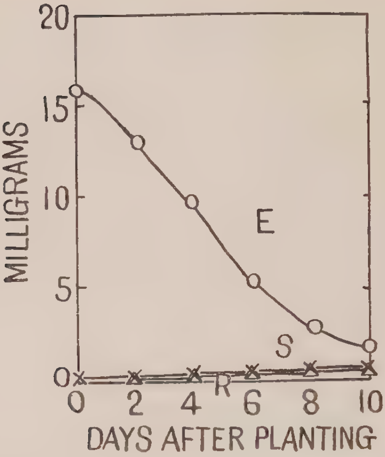

Fig. 2. Changes in the starch content of embryonic organs of rice during germination.

E: endosperm.

S: shoot.

R: root.

Values per one individual are plotted. Analytical method is refered from (*8*).

tration of the starch samples taken in the course of germination was carried out. Fig. 4 shows the typical results obtained from the starch samples from the non-germinated and of the 6th-day after planting. The inflection point of the latter curve shifts distinctly towards the higher content of iodine solution. From these and other data for the samples of the 2nd- and the 4th-day after planting, the iodine affinities of the starch in the course of germination were calculated and shown in Table II.

It is seen that the iodine affinity increases as the germination proceeds. This is compatible with the result of the blue values. Measurements of the iodine affinity on the starch samples from glutinous rice were also made, but they were close to the blank curve regardless the germination stage.

DISCUSSION

A probable explanation of the increase in *relative* amylose content

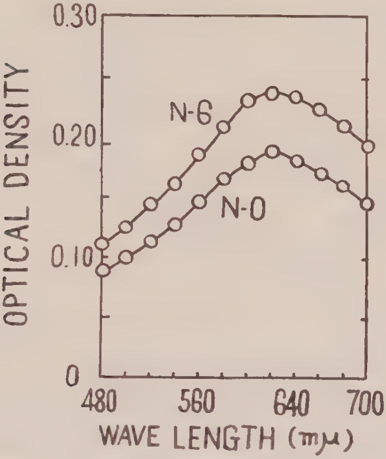

FIG. 3. Absorption curves of the starch samples in the endosperms of rice before and after germination.

N-O: zero time control.

N-6: 6 days germination.

The solutions contained 5 mg. of starch, 10 mg. of iodine, and 100 mg. mg. of potassium iodide in 200 ml.

of starches isolated from the germinating seeds is that the amylopetcin component would be digested more easily than the amylose component.

Are there any special enzymes which will digest amylopectin preferably Meyer (*11*) has reported that soluble starch is hydrolysed more rapidly than pure amylose by beta-amylase *in vitro*, because the former has a much more reducing ends than the amylose component in the equivalent molecular concentration. However, the greminting cereal grains contain rather active alpha-amylase besides beta-amylase (*12*). So that this could not be adopted to explain our present phenomenon.

Another possibility of the special enzyme is the action of isoamylase or R-enzyme. These enzymes (probably the both may be the same) attack the 1,6-branching point of the amylopectin and produce the amylose component from the amylopectin.

The existence of this enzyme in mature rice grain has been reported by Minagawa (*13*), and Miyake and Hayashi (*14*) about twenty years ago though the enzyme was supposed as the starch synthesizing enzyme, " amylosynthease ", at that time. However, this enzyme in

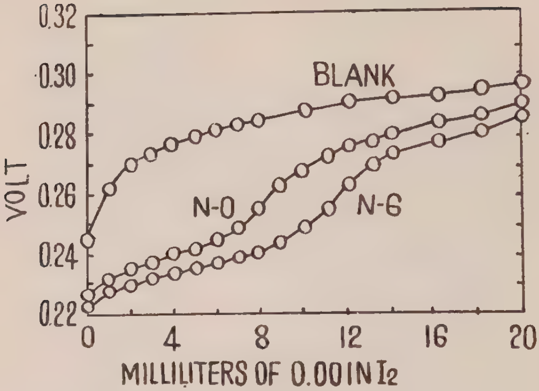

Fig. 4. Potentiometric titration curves of the starch samples in the Endosperms of rice before and after germination.

N-O: zero time control.

N-6: 6 days germination.

Samples contined 40 mg. of starch. As analytical method, that given in Ref. (*9*, *10*) was followed.

rice grain has not been studied further and it is questionable whether it participates in the degradation of starch during germination or not.

We would rather consider that the amylose component in the germinating rice grain is digested difficultly by the starch degrading enzymes because its physical properties are less suitable for digestion as compared with that of the amylopectin.

An electronmicroscopic observations made by one of the authors (Z. N.)6) indicated that the starch granule would be consisted of very small spheres of amylose and amylopectin micelles. The amylose micelle would form more compact spheres than amylopectin by its strong intra- and intermolecular affinity, and so would be more resistant against the degradative enzymes.

Recently Badenhuizen (*15*) reported the existence of the nuclei which are stained blue with iodine in the starch granule of some plant sources after treatment with dilute hydrochloric acid. It would give some support to our present consideration.

SUMMARY

Blue value and iodine affinity were measured on the various starch

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6) Z. Nikuni, unpublished result.

Table I

Changes in Blue Value of the Starch Samples in the Endosperm during Germination

(*a*) *Non-Glutinous Rice*

Days after planting	0	2	4	6
Blue value of non-defatted samples	0.128	0.121	0.150	0.164
Blue value of defatted samples	0.139	0.161	0.183	0.189

(*b*) *Glutinous Rice*

Days after planting	0	2	4	6
Blue value of non-defatted samples	0.008	0.009	0.012	0.015
Blue value of defatted samples	0.023	0.023	0.026	0.034

The method of McCready *et al.* (*8*) was applied to determine the starch contents and blue values. Blue value was tentatively defined as described in *Footnote 4.*

Table II

Changes in Iodine Affinity of the Starch Samples in the Endosperm of Rice during Germination

Days after planting	0	2	4	6
Iodine affinity (%)	3.3	3.3	4.0	4.4

Iodine affinity was given as the percentages of the bound iodine per starch (*w./w.*). Analytical methods arc refered from (*9*, *10*).

specimens from the endosperms of glutinous and non-glutinous rice seeds in the course of germination, and found that the relative amylose content of starch in the endosperms increases as degradation of the starch proceeds. Some probable reasons are discussed.

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THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

VI. SPHINGOLIPIDS OF ERYTHROCYTES WITH RESPECT TO BLOOD GROUP ACTIVITIES*

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It has previously shown that globoside, a glycolipid isolated from human red blood cells, possesses the capacity to inhibit hemagglutination of erythrocytes of a given blood group by its corresponding isoantibody (*1*).

At that time, the specimen of globoside was prepared from the erythrocytes pool irrespective of blood group, so the inhibitory activity revealed by the lipid was ubiquitous for all blood groups.

On the other hand, the effort to elucidate the chemical properties and structure of individual blood group substance, especially to discover the differences between them in relation to their immunological specificity, has been continued in several laboratories, and a number of excellent findings have been reported.

The group materials used by those workers are obtained mainly from tissue fluids or secretions such as gastric mucin, pseudomucinous ovarian cyst fluid, saliva, urine, meconium, commercial pepsin, *etc.*, and the active preparations obtained from these sources have been found to be largely polysaccharide in nature but to contain about 22–25 per cent of amino acids. The polysaccharide consists of D-galactose, L-fucose, acetyl-glucosamine and acetyl-chondrosamine. Though these purified specimens were homogeneous by several physico-chemical methods, they could possibly contain more or less inert materials and the differences if detected might probably be due to the moiety other than group active site.

Globoside has been reported to have an approximate structure of lignoceryl-(or nervonyl) sphingosine acetylchondrosamine trigalactoside by us (*2*) and -tri or -tetra hexoside by Klenk and Lauenstein (*3*) and is assumed to be highly polymerized in aqueous solution (*4*).

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* This work was read at the 26th General Meeting of Japanese Biochemical Society held in Sendai, in April, 1954.

In this report globoside and sphingomyelin samples were obtained from erythrocytes pool of individual blood group A, B, O and AB, and several chemical examinations were studied to find out their structural differences.

EXPERIMENTAL, RESULTS AND DISCUSSION

Preparation of Materials—For the preparation of individual group active globoside, each 20 l. of fresh human red blood cells belonging to group A, B, O and AB were generously supplied by Dr. Kuniyuki of Nihon Seiyaku Co.

The preparative method was almost the similar to the procedure reported in the 3rd paper of this series (*2*).

The crude globoside thus obtained was extracted repeatedly with hot 95 per cent acetone until the hexosamine content reached its maximum value.

Finally, the insoluble colorless powder was recrystallized from hot methanol. Yield, 200–400 mg.

Analytical Constants—For the simplification, the analytical data are summarized in Table I.

TABLE I

Analytical Constants of Globoside with Respect to Blood Group Activities

	Globoside A	Globoside B	Globoside O	Globoside AB
	%	%	%	%
C	62.70	61.77	61.24	62.97
H	9.64	9.41	9.37	9.49
N	2.27	2.16	2.08	2.32
P	0.064	0.012	0.1	0.1
$[\alpha]_D$ (in pyridine)	+13.2°	+13.8°	+13.5°	+13.1°
Hexosamine (as HCl salt)	9.66	9.34	13.47	14.57
Reducing value (as galactose)	45.94	53.75	50.37	57.40

As shown in Table I, there are no great differences with all four groups, therefore, it seems almost impossible to find out any paralellism between group activity and these analytical values.

Hemagglutination Inhibition—Diminishing concentrations of the preparations under test (0.25 ml.) were mixed with 0.25 ml. of a human anti-A (alpha), anti-B (beta) serum or anti-O fowl serum (absorbed by ABe cells) representing in amount 2 completely agglutinating doses, and allowed to stand at 37° for 1 hour. Freshly drawn A, B or O erythrocytes (0.1 per cent in 0.85 per cent NaCl) were then added (0.5 ml.). The mixtures were thoroughly shaken, set aside for 2 hours at 37° and then examined for isoagglutionation. The results are shown in Table II.

TABLE II

Isoagglutination Inhibition of Globosides

		1:2 $\times 10^3$	1:4 $\times 10^3$	1:8 $\times 10^3$	1:16 $\times 10^3$	1:32 $\times 10^3$	1:64 $\times 10^3$	1:128 $\times 10^3$	1:256 $\times 10^3$	1:512 $\times 10^3$	1:1024 $\times 10^3$
Gl. A	A-α	−	−	−	−	−	−	−	−	−	+
	B-β	+	+	+	+	+	+	+	+	+	+
	O-o′	±	±	±	+	+	+	+	+	+	+
Gl. B	A-α	±	+	+	+	+	+	+	+	+	+
	B-β	−	−	−	−	−	−	−	−	±	+
	O-o′	−	−	−	±	+	+	+	+	+	+
Gl. AB	A-α	−	−	−	−	−	−	−	−	−	+
	B-β	−	−	−	−	−	+	+	+	+	+
	O-o′	−	−	−	−	±	+	+	+	+	+
Gl. O	A-α	±	+	+	+	+	+	+	+	+	+
	B-β	+	+	+	+	+	+	+	+	+	+
	O-o′	−	−	−	−	−	−	−	−	±	±

Addendum—Hog and equine stroma glycolipids showed no inhibitory activity at 0.1 per cent concentration. According to the experiment of Dr. K. Tsuge, there is no correlation between globoside and MN or Q blood group activity.

pH-Stability Range of Blood Group Substances—Three series of solutions of globoside A, B together with group A material isolated from hog gastric mucoid* were prepared by the addition of 0.5 ml. of buffer solutions varying in pH from 1.45 to 12.1 to the 0.5 ml. of diminishing concentrations of group substances in saline. After mixing, the whole set were heated in a water bath at 100° for 2 hours.

Each solution was then assayed for activity by hemagglutination inhibition. The results were read after placing overnight in an ice-box and are given in Tables III, IV and V.

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* Kindly supplied by Dr. Z. Yoshizawa of Prof. Masamune's laboratory.

TABLE III

Stability of Globoside A to Various pH

pH / Inhibitor conc.	1:16 $\times 10^3$	1:32 $\times 10^3$	1:64 $\times 10^3$	1:128 $\times 10^3$	1:256 $\times 10^3$	1:512 $\times 10^3$	1:1024 $\times 10^3$	1:2048 $\times 10^3$
Unheated control	−	−	−	−	−	−	−	+
1.45	−	−	−	−	+	+	+	+
2.1	−	−	−	−	−	−	−	+
4.4	−	−	−	−	−	−	−	+
7.1	−	−	−	−	−	−	−	+
9.1	−	−	−	−	−	−	−	+
9.7	−	−	−	−	−	−	−	+
12.1	−	−	−	±	±	±	±	+

TABLE IV

Stability of Globoside B to Various pH

pH / Inhibitor conc.	1:16 $\times 10^3$	1:32 $\times 10^3$	1:64 $\times 10^3$	1:128 $\times 10^3$	1:256 $\times 10^3$	1:512 $\times 10^3$	1:1024 $\times 10^3$	1:2048 $\times 10^3$
Unheated control	−	−	−	−	−	+	+	+
1.45	−	−	+	+	+	+	+	+
2.1	−	−	−	−	−	+	+	+
4.4	−	−	−	−	±	+	+	+
7.1	−	−	−	−	−	+	+	+
9.1	−	−	−	±	+	+	+	+
9.7	−	−	−	±	+	+	+	+
12.1	−	−	±	±	+	+	+	+

The data in Tables III, IV and V indicate that globoside A and B samples are more stable than group mucoid as measured by hemagglutination inhibition. In general, no significant loss of activity appeared when the pH was between 2.1 to 9.7. Partial destruction resulted in case of globoside from exposure to a pH 1.45 or 12.1 and practically complete loss of activity occurred at these pH with mucoid substance.

TABLE V

Stability of Group Mucoid to Various pH

pH \ Inhibitor conc.	1:16 ×10^3	1:32 ×10^3	1:64 ×10^3	1:128 ×10^3	1:256 ×10^3	1:512 ×10^3	1:1024 ×10^3	1:2048 ×10^3	1:4096 ×10^3
Unheated control	−	−	−	−	−	−	−	−	±
1.45	+	+	+	+	+	+	+	+	+
2.1	−	−	−	−	−	−	+	+	+
4.4	−	−	−	−	−	−	−	±	±
7.1	−	−	−	−	−	−	−	−	+
9.1	−	−	−	−	−	−	−	−	+
9.7	−	−	−	−	−	−	±	+	+
12.1	+	+	+	+	+	+	+	+	+

Acid Hydrolysis of the Group-specific Substances—Globosides and group active mucoid were hydrolysed in glass-stoppered vessels at 100° with (a) *N* acetic acid, (b) 0.05 *N* HCl and (c) 0.5 *N* HCl. After heating for the appropriate length of time, the vessels were cooled, opened and the contents were exactly neutralised with NaOH, phenolphthalein as an indicator. The neutral solution was divided into three portions, the first was used for determination of reducing value, the second for free hexosamine and isoagglutination inhibition was assayed with the third portion.

The results obtained were indicated in Figs. 1, 2 and 3.

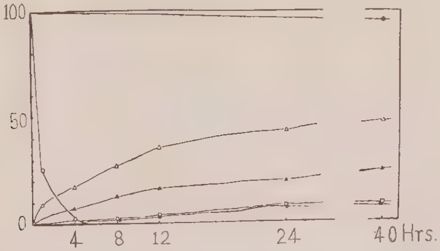

FIG. 1. Effect of hydrolysis with *N* acetic acid on the activity of isoagglutination, ordinate: per cent serological activity, reducing value and free hexosamine, abscissa: heating time.

Hog gastric mucoid, —○—○— serological activity, —△—△— reducing value, —□—□— hexosamine.

Globoside A, —●—●— serological activity, —▲—▲— reducing value, —■—■— hexosamine.

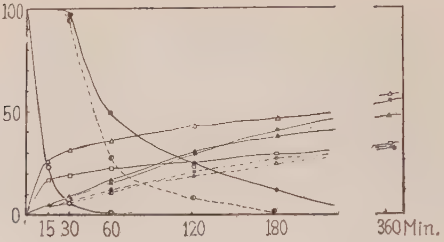

FIG. 2. Effect of hydrolysis with 0.05 *N* HCl on the activity of isoagglutination, ordinate: per cent serological activity, reducing value and free hexosamine, abscissa: heating time.

Hog gastric mucoid, —○—○— serological activity, —△—△— reducing value, —□—□— hexosamine.

Globoside A, —●—●— serological activity, —▲—▲— reducing value, —■—■— hexosamine.

Globoside B, —◐—◐— serological activity, —◭—◭— reducing value, —◧- -◧— hexosamine.

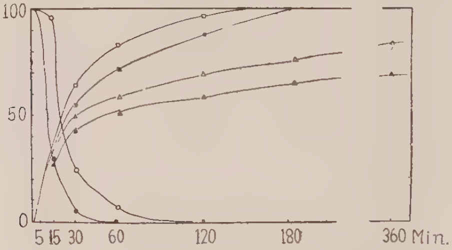

FIG. 3. Effect of hydrolysis with 0.5 *N* HCl on the activity of isoagglutination, ordinate: per cent serological activity, reducing value and free hexosamine, abscissa: heating time.

Globoside A, —○—○— serological activity, —△—△— reducing value, —□—□— hexosamine.

Globoside B, —●—●— serological activity, —▲—▲— reducing value, —■—■— hexosamine.

As shown in Fig. 1, during treatment with *N* acetic acid at 100°, the group material isolated from hog gastric mucin rapidly lost its capacity to inhibit isoagglutination in a few hours, whereas under the same condition the activity of globoside remained intact; thus, the more stable nature of globoside as compared with group mucoid was again observed.

The Ratio of Galactose to Glucose—As shown previously (*2*), a trace of glucose was detected besides galactose by paper chromatography and the proportion of these two sugars was determined with carbazole-sulfuric acid technique. In this time, the similar experiments were carried out to know whether the difference of group activity of globosides might be represented by these ratios. As shown in Fig. 4, in all four instances, the amounts of glucose were extremely smaller as compared with that of galactose and the ratios were not appreciablly different with each other.

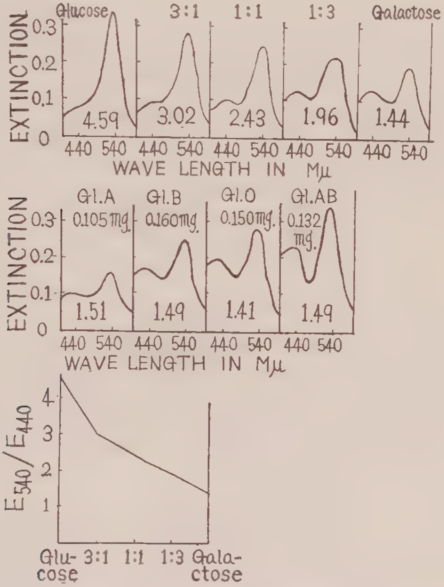

FIG. 4. The ratio of galactose to glucose of globoside A, B, O and AB by carbazole reaction. The figure indicates E_{540}/E_{440}.

The Proportion of Chondrosamine to Glucosamine—It has previously been reported by Klenk and Lauenstein (*3*) and by two of the present authors (*2*), only chondrosamine was detected paperchromatographically as the component hexosamine of human red cells glycolipid or globoside. However, a small amount of glucosamine could be separated from chondrosamine with the column chromatography using cation exchanger devised by Gardell (*5*).

Purified globoside samples (3–5 mg.) were hydrolysed with 2*N* HCl at 100° for

4 hours, filtered and evaporated to dryness. The residue was taken up in 0.5 ml. of 0.3 *N* HCl and chromatographed through the Dowex 50 column (40 cm. × 0.6 cm.) at a flow rate of about 1.5 ml. per an hour. The effluent was collected in 1 ml. by a fraction collector. The amount of hexosamine in each tube was estimated by Gardell's procedure. The results obtained are shown in Fig. 5.

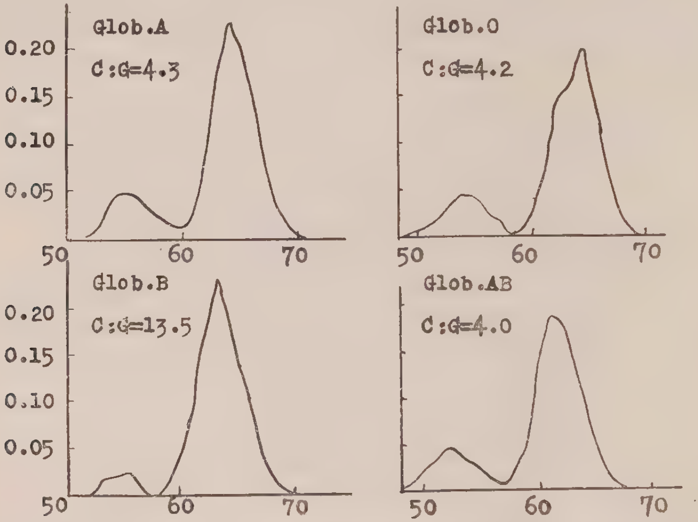

FIG. 5. The nature of hexosamine in globoside, ordinate: extinction, abscissa: effluent volume (ml.) The former peak: glucosamine, the latter: chondrosamine.

The proportion of chondrosamine to glucosamine in globoside is somewhat peculiar with blood group. The ratios are as follows: Globoside A 4.3 ; globoside B 13.5 ; globoside O 4.2 ; globoside AB 4.0.

Only in case of Globoside B, the ratio is considerably higher than the others. The determination duplicated with another Globoside B specimen prepared from entirely another pool of group B erythrocytes resulted the ratio was 11.9.

Recently, Leskowitz and Kabat (*6*) developed a method for the separation and estimation of these two hexosamines as the 2,4-dinitrophenylaminohexitols. Using this technique, they reported hog and human group A substances were found to have the glucosamine: chondrosamine ratios averaging 1.5 and 1.6, respectively ; group O(H) hog and human preparations 2.2 and 2.5, and human B preparations

2.8. Independently, Rondle (*7*) and Rondle and Morgan (*8*) also reported some correlation between serological specificity and hexosamine ratio. Group A mucoids usually give a glucosamine:chondrosamine ratio from 1.0 to 1.5, whereas the ratio is closer to 3 for B and Le^a substances and varies from 3 to 12 for H substances. Most recently, Yoshizawa (*9*) reported the ratio 1.3–1.5 for A, 2.4–2.6 for O and 1.6–1.7 for AO group mucoid. As shown in these figures, group mucoids contain more glucosamine than chondrosamine just reversely to the case of globoside.

On the other hand, 90 per cent phenol extract of defatted human blood stroma (so-called polysaccharide fraction, (1)), which possesses almost no capacity to inhibit isoagglutination but a potential inhibitory activity against influenza virus hemagglutination, also contained hexosamine and hemataminic acid (in the form of *N*-acetyl-prehemataminic acid, unpublished). By the chromatography, it proved the glucosamine content of this fraction is more than chondrosamine contrary to lipid-hexosamine. (Fig. 6).

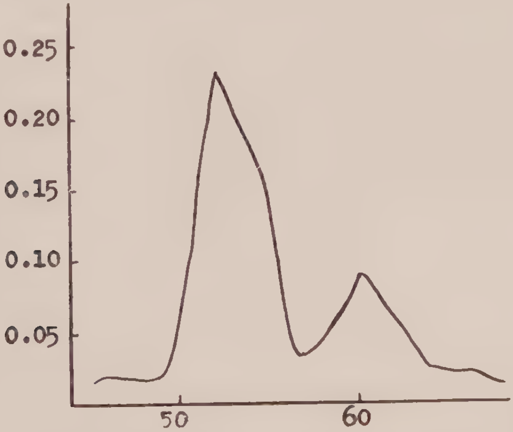

Fig. 6. Chromatographic separation of hexosamine in human stroma polysaccharide, ordinate: extinction, abscissa: effluent volume (ml). The former peak: glucosamine, the latter: chondrosamine. Glucosamine: chondrosamine, 3.3.

Sphingomyelin of Human Erythrocytes

Purification of Sphingomyelin—Sphingomyelin was purified by the procedures previously described (*10*).

A pyridine-insoluble material obtained from the ether-insoluble portion of the ether-methanol extract from human blood stroma, which appeared waxy was named *Sph. 1.*

Sph. 1. was treated with glacial acetic acid by the method of Thannhauser, *et al.* (*11*), (*Sph. 2.*).

Sph. 2. was taken up in ethanol-benzin (1:9) and passed through a column of alumina. A material obtained from the eluate was called *Sph. 3.*

Sph. 3. was dissolved in ethanol-glacial acetic acid (9:1), further purified by passing through an alumina column and a colorless powder thus obtained was designated as *Sph. 4.*

Sph. 4. was recrystallised from ethyl acetate and a fine colorless powder was obtained (*Sph. 5.*).

The analyses and yields of the sphingomyelin at various stages of purification described above are shown in Table VI.

Sph. 5. is easily soluble in benzene, chloroform, soluble in warm ethanol, warm pyridine and hot ethyl acetate. Heated in a capillary tube, it turned yellow at 180° and melted with brown colorlation at 204°. 0.5013 g. of the substance dissolved in chloroform-methanol (1:1) to 10 ml. gave, in a 1 dm. tube, $\alpha_D = +0.286°$, hence $[\alpha]^{15}_{D} = +5.70°$.

TABLE VI

Analysis and Yield of Sphingomyelin from Human Blood Stroma

	C	H	N	P	Glycerol	Sugar	Yield
	%	%	%	%	%	%	g.
Sph. 1.	......	......	3.07	3.60	......	0.95	9.25
Sph. 2.	......	......	3.13	3.70	......	0.71	6.80
Sph. 3.	......	......	3.43	3.69	......	0.51	5.95
Sph. 4.	......	......	3.31	3.50	0.75	0.30	4.5
Sph. 5.	67.64	11.19	3.29	3.57	0.70	0.03	......

Hydrolysis of Sphingomyelin—6 g. of purified sphingomyelin was added to 100 ml. of 5 per cent methanolic hydrochloric acid in a sealed tube and heated in a boiling water bath for 3 hours. After cooling, the reaction mixture was extracted three times with petroleum ether. The extract was washed with water, dried over sodium sulfate and evaporated to dryness. Methyl ester of fatty acid thus obtained weighed 2.311 g. (38.5 per cent). The methanolic solution of reaction mixture after extraction of the ester was made alkaline with baryt water. After removal of barium sulfate, the solution was extracted three times with ether. From this ethereal extract, light brown-colored free base was obtained, weighing 1.46 g.

Fractionation of Fatty Acid—The methyl ester (1.793 g.) was recrystallised from methanol to colorless plates, melting at 56–7° (591 mg.). It was converted to free acid melting at 80°, and showed no depression of the melting point when fused with the authentic sample of lignoceric acid, melting at 83°.

From the mother liqor of methyl lignocerate, a solid material (1.15 g.) was obtained after evaporation of methanol and was fractionated by vacuum distillation; the results obtained are shown in Table VII.

TABLE VII

Fractional Distillation of Fatty Acids in Human Stroma Sphingomyelin

	Methyl ester of fatty acids				Free fatty acids		
	B.p.	M.p.	I.N.	Yield	M.p.	I.N.	COOH
				%			%
Fr. 1		57–8°		33			11.99
Fr. 2	123–7°/0.25–0.3	25–6°	4.23	17.5			18.04
Fr. 3	149–155°/0.25		25.99	18		25.33	
Fr. 4	155–170°/0.25		57.53	15.5	40–1°	66.46	11.63
Fr. 5	170–186°/0.25		47.99	6	40–1.5°	51.70	
Fr. 6	Residue			9.5			

In Table VII, Fr. 1. is lignoceric acid, Fr. 4+5 correspond to nervonic acid and Fr. 2+3 are mainly palmitic acid. This data was confirmed by reversed-phase column chromatography as shown in the successive paper (*12*).

Identification of Base Fraction—The base fraction was dissolved in warm petroleum ether. The solid separated on cooling was acetylated with acetic anhydride and pyridine. Acetate, thus obtained, was recrystallised from acetone to colorless needles, melting at 100–101°.

Analysis—

Calc. for triacetylsphingosine, $C_{24}H_{43}NO_5$
C 67.73, H 10.18, N 3.29
Found: C 67.65, H 9.76, N 3.42

Sphingomyelin of Individual Group—Sphingomyelin samples were obtained as the by-products of group-specific globosides. Analytical constants of each purified sphingomyelin are given in Table VIII. No appreciable difference due to blood group is found as shown in the Table.

By reversed phase chromatography, the nature and amount of component fatty acids of individual sphingomyelin were examined. However, the chromatographic pattern was in every case almost the same.

SUMMARY

1. The isolation and characterization of globoside samples which

TABLE VIII

Analytical Constants of Sphingomyelin A, B, O and AB

	Sphingomyelin A	Sphingomyelin B	Sphingomyelin O	Sphingomyelin AB
C %	68.63	68.34	68.76	68.13
H %	11.39	11.24	11.39	11.55
N %	3.43	3.68	3.48	3.58
P %	3.72	3.81	3·76	3.60
$[\alpha]_D$ in $CHCl_3$-CH_3OH	+ 7.22°	+ 5.67°	+ 7.22°	+ 6.17°
Reducing value (as galactose) %	0.27	0.69	1.03	0.90
Glycerol %	0.39	0.09	0.32	0.27

are responsible for the ABO-system blood group specificity are described.

2. The ratios of hexosamine components appear to show some correlation with specificity.

3. On mild acid hydrolysis, group activity exhibited by globoside is much more stable than group active mucoids.

4. Sphingomyelin obtained from erythrocytes of all four groups did not show any chemical differences.

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THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

VII. STUDIES ON CHONDROSAMINE-CONTAINING GLYCOLIPID AND SPHINGOMYELIN OF HOG BLOOD STROMA

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In the previous studies of this series, both a hexosamine-containing glycolipid designated as ' globoside ' and a hemataminic acid (or neuraminic acid)-containing one named ' hematoside ' were isolated from stroma of human (*1*) and horse (*2*) erythrocytes, respectively. Similar results were obtained by Klenk *et al.* in the studies on ganglioside in human brain (*3*) and bovine spleen (*4*), as well as on lipids in erythrocyte stroma of human beings (*5, 6*), cattle (*6*) and horse (*7*). Quantitative analysis of the amounts of lipid-hexosamine and lipid-hemataminic acid in erythrocyte stroma of various species of animals (*8*) suggested that animals could be classified into four groups with respect to the distribution of hexosamine and hemataminic acid. In that paper, the total lipid in stroma of hog erythrocytes was reported to contain a considerably large quantity of hexosamine, whereas the amounts of hemataminic acid contained was to be null or few if it exists. However, the results of the colorimetric estimation of hemataminic acid in small scale, at that time, was uncertain, because the resulted color tone was differed from that of characteristic Bial's reaction. Therefore, a further detailed study in a large scale was desired.

The present study revealed that glycolipid isolated from stroma of hog erythrocytes gave no coloration of hemataminic acid with Bial's orcinol reagent, while it contained a large amount of hexosamine. This fact indicates that the glycolipid in hog erythrocyte stroma is ' globoside ' type as observed in human, sheep, or goat erythrocytes. This glycolipid was purified in a form of colorless powder (H_{261}), which exhibited a high reducing value and contained a large amount of hexosamine.

Further detailed analysis of this substance was conducted, and the results were compared with those of globoside and Klenk's glycolipid.

Sphingomyelin found in the stroma accompanied with the above glycolipid was also purified and analyzed.

EXPERIMENTAL

Analytical Methods—Phosphorus was estimated by Allen's method (*9*), iodine number by Yasuda's (*10*) and hexosamine by Blix's (*11*). Reducing value was determined cerimetrically by the method of Brand and Sperry (*12*). Dry combustion methods were used for the estimation of C, H and N.

Preparation of Material—34 *l.* of fresh hog blood corpuscles, separated from plasma by centrifugation, was treated in the same manner as described in the previous paper (*1*) and 290 *g.* of dried stroma was obtained.

Extraction of Lipids—The procedures described in the first report (*2*) were followed with slight modifications. Ether-methanol extracts from 280 g. of the stroma was concentrated *in vacuo,* to which acetone was added. The resulting precipitate was separated by decantation of supernatant fluid, then dissolved in a small amount of anhydrous ether and kept in a refrigerator. A precipitate appeared was separated by centrifugation and washed several times with ether. This precipitate was white waxy powder which mainly consisted of a mixture of sphingomyelin and glycolipid. Yield was 11 g. It contained 2.59 per cent phosphorus, 2.20 per cent hexosamine and gave positive Molisch's reaction.

Separation of Glycolipid and Sphingomyelin—The material described above was dissolved in warm pyridine and the solution was cooled in an ice-box. The resulting precipitate was separated by centrifugation. This procedure was repeated once more. The final precipitate was washed with acetone and ether successively. White powder thus obtained weighed 3.4 g. (crude sphingomyelin), and contained 3.12 per cent phosphorus. The supernatant pyridine solution was evaporated nearly to dryness under reduced pressure and acetone was added. The resulting precipitate was washed with ether. Yellowish powder (H_1) thus obtained weighed 1.75 g. and contained 1.01 per cent phosphorus, 31.3 per cent sugar and 6.6 per cent hexosamine. This material showed negative Bial's orcinol reaction.

Purification of the Glycolipid—The pyridine soluble fraction, H_1, was treated twice more with pyridine and a small amount of precipitate was separated by centrifugation. The resulting precipitate, crude sphingomyelin, weighed 0.5 g. and contained 1.21 per cent phosphorus. The supernatant pyridine solution was passed through an alumina column and the eluate was evaporated *in vacuo*. White powder (H_2) was precipitated by addition of acetone to the concentrated eluate. This material (1.65 g.) contained 0.10 per cent phosphorus, 11.84 per cent hexosamine and 36.5 per cent reducing value. 1.60 g. of the fraction H_2 was extracted with various volumes (from 100 ml. to 160 ml.) of 95 per cent aqueous acetone in a boiling water-bath successively. Each sediment which separated out from the extracts on cooling was named H_{21}, H_{22}, —H_{26} in that order, and the insoluble residue was designated as H_{26}. Another residue which was obtained on evaporation of the combined supernatant was designated as H_{27}. The analyses of these fractions are shown in Table I.

TABLE I

Analyses of the Glycolipid Fractions from Hog Erythrocyte Stroma

	Extraction time and volume of 95% acetone	Yield (from 1.6 g. of H_2)	Sugar	Hexosamine	C	H	N	P
		g.	%	%	%	%	%	%
H_{21}	15 min., 160 ml.	0.22	38.02	5.57	62.51	9.74	2.39	0.04
H_{22}	30 min., 150 ml.	0.22	38.46	10.48			2.16	
H_{23}	1 hr., 130 ml.	0.18	39.02	10.28			2.32	
H_{24}	2 hrs., 110 ml.	0.14	40.25	12.23	61.79	9.64	2.49	
H_{25}	4 hrs., 100 ml.	0.13	41.37	12.04	60.15	9.72	2.02–2.15	
H_{26}		0.59	43.55	12.92	60.36	9.16	2.02–2.36	0.05
H_{27}		0.08	32.96	6.74				

H_{26} was recrystallized twice from methanol and the colorless friable powder obtained was named H_{261}. This material melted at 195–200° with decomposition and was somewhat hygroscopic. The residual filtrate was evaporated *in vacuo* to a syrup, to which acetone was added. The resulting precipitate was separated by centrifugation, washed with ether and dried *in vacuo*. This fraction was designated as H_{262}.

Analysis—

H_{261}: C 60.16, 60.20; H 9.60, 9.42; N 2.07, 2.31. Sugar (as galactose) 49.4–53.8; Hexosamine (as glucosamine-HCl) 12.6–13.3; Acetyl 7.53–8.50 (after hydrolysis with 50 per cent sulfuric acid for 1 hour), 4.99 (after hydrolysis with the same reagent for 3 hours), 2.76–3.76 (after hydrolysis with 5N-sodium hydroxide for 3 hours). $[\alpha]_D^{20°} = +21.8°$ (68.510 mg. in 2 ml. of pyridine).

H_{262}: Sugar (as galactose) 49.8; Hexosamine (as glucosamine-HCl) 13.7; N 2.05.

Hydrolysis of H_{261}

200 mg. of H_{261} was added to 80 ml. of 3N-hydrochloric acid and heated in a boiling water-bath for 3.5 hours. The reaction mixture was at first faintly opalescent, and then a small amount of insoluble material appeared. The white precipitate which separated our after cooling was filtered by suction, washed once with distilled water and dried in an evacuated desiccator over potassium hydroxide. This material (I) included a mixture of fatty acids and hydrochlorides of sphingosine or sphingosine-like base, and the filtrate (II) contained sugar fractions and trace of basic material.

Fatty Acid Fraction—(I) was dissolved in approximately 20 volumes of 5 per cent methanolic sulfuric acid and refluxed for 1 hour in a boiling water-bath. After cooling, the content was extracted 3 times with petroleum ether, and the extract, after washing with water, was dried over sodium sulfate, filtered, and evaporated to dryness. The residual methyl ester of fatty acids solidified at room temperature, and weighed 46 mg. (23 per cent). The substance was recrystallized from methanol to colorless plates (42 mg.) melting at 56–57°, which was then converted to free acid by saponification with methanolic potassium hydroxide. The free acid was recrystallized to colorless plates from petroleum ether, melting at 80°. It showed no depression of the melting point when mixed with an authentic sample of lignoceric acid (m.p. 82°).

From the mother liquor of methyl lignocerate, after evaporation of methanol, only 3 mg. of solid ester was obtained. The hydrolysis of this material was not attempted.

Base Fraction—After extraction of the ester fraction with petroleum ether, methanol was evaporated *in vacuo*. The residue was made alkaline with baryt to phenolphthalein, then the resulting precipitate of barium sulfate was removed by filtration and washed with ether. The filtrate was extracted with each 50 ml. of ether-petroleum ether mixed in varying ratio of 1:3, 1:1, and 3:1, successively. These extracts were combined, washed with water, filtered after drying over sodium sulfate, and evaporated to dryness, The faintly brown colored crude base thus obtained weighed 29 mg. (15 per cent). This solid, which was readily soluble in petroleum ether, was dissolved in acetone, and alcoholic sulfuric acid was carefully added after removal of a little amount of insoluble material. The resulting white precipitate was centrifuged, washed with cold alcohol, and dried *in vacuo* over phosphorus pentoxide. Its amount was too small for detailed analysis and only the estimation of nitrogen was carried out giving the result of 3.72 per cent. This figure is approximately equal to that of methoxy-sphingosine-sulfate, 3.86 per cent.

Sugar Fraction—Fraction II was evaporated under reduced pressure. To the residue, water was added and concentrated *in vacuo* to remove hydrochloric acid as much as possible. This process was repeated several times, and a small quantity of saturated silver sulfate solution was added to the concentrate to remove the remaining trace of chloride. After separation of the silver chloride by filtration, the filtrate was made alkaline with barium hydroxide, and a precipitate which appeared was filtered off. Then the filtrate was extracted several times with ether. However, the ether extracts contained only a trace of material. The aqueous solution was then saturated with hydrogen sulfide and then with carbon dioxide to remove silver and barium ions. The filtrate was passed through a column of cation exchanger (Dowex 50) for the separation of hexosamine from neutral sugars. The column was washed with water and the eluate was concentrated *in vacuo* to about 2 ml. This concentrate (III) contained neutral sugars, which were identified by paper chromatography and by preparation of the sugar derivatives. The column was then eluted with a large amount of *N* hydrochloric acid (about 500 ml) and concentrated *in vacuo* (IV). Fraction IV showed an intense color reaction of hexosamine by the method of Elson and Morgan. To the fraction III was added 0.1 g. of freshly distilled α-methylphenylhydrazine. Light yellow crystals which appeared after cooling overnight in a refrigerator, was separated, and recrystallized from methanol to colorless plates, melting at 188–189°. It showed no depression of melting point with pure D-galactose-α-methylphenylhydrazone, melting at 188–189°.

Analysis—

Calculated for $C_{13}H_{20}O_5N_2$: C 54.92, H 7.09, N 9.85
Found: C 54.77, H 7.21, N 9.73

Paper Chromatography of Sugars

Neutral Sugar—Portions of 1 per cent glucose, galactose solution and above mentioned sugar component of H_{261} were chromatographed on Tōyō Filter Paper No. 50 using a developing solvent system of a mixture of water-pyridine-ethyl acetate (2:1:2). The sheet was developed for 18 hours, and positions of these sugars were indicated by aniline-hydrogen-phthalate. The results are shown in Fig. 1.

Hexosamine—About 4 mg. of H_{261} was hydrolyzed with $3N$ hydrochloric acid, and then dried over potassium hydroxide in an evacuated desiccator. 0.2 ml. of water was added and the solution was chromatographed on Tōyō Filter Paper No. 50, using collidine saturated with water as a developing solvent system, simultaneously appropriate amounts of glucosamine- and chondrosamine-hydrochloride were placed as controls. The paper was developed for 40 hours, and the spots were detected by a method of Elson and Morgan modified for paper chromatography. The results are shown in Fig. 2.

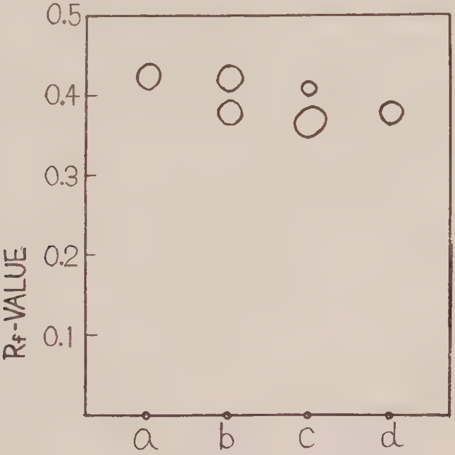

FIG. 1. Paper chromatogram of neutral sugars:
(a) glucose, (b) mixture of glucose and galactose, (c) sugar mixture of H_{261}, (d) galactose.

Column Chromatography of Hexosamines—The procedure employed in the present study was essentially the same with the method of Gardell (*13*), and since its detail

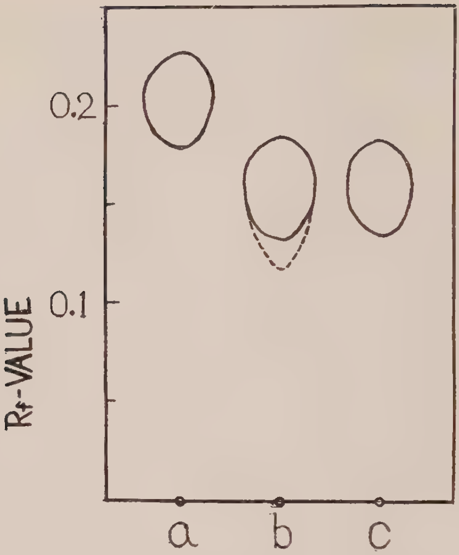

FIG. 2. Paper chromatogram of hexosamines:
(a) glucosamine-HCl, (b) hydrolysate of H_{261}, (c) chondrosamine-HCl.

will be described in the next paper (*14*), only the results are shown in Fig. 3.

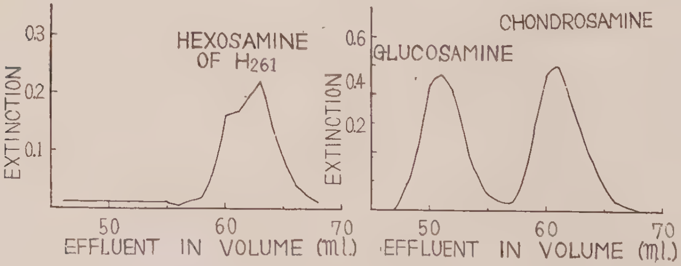

FIG. 3. Separation of glucosamine and chondrosamine on Dowex 50 ion exchange resin.

Purification of the Hog Sphingomyelin—The sphingomyelin of hog erythrocyte stroma was purified by the method approximately the same as that for human or equine sphingomyelin described in the previous papers (*2*, *15*). The analyses of the purified hog sphingomyelin are as follows.

C 67.22, H 11.78, N 3.66, P 3.71
$[\alpha]_D^{18°}=+7.3°$ (80.529 mg. in 2 ml. chloroform-methanol, 1:1)

Hydrolysis of the Sphingomyelin—The sphingomyelin was hydrolyzed with 5 per cent methanolic hydrochloric acid in a sealed tube on a boiling water-bath for 2 hours. Resulting ester fraction of fatty acid as component of the sphingomyelin was extracted with petroleum ether three times from the reaction mixtures. 1.148 g. of the ester was obtained from 3 g. of the sphingomyelin (38.3 per cent). The residual hydrolysate, after extraction of fatty acid methyl esters, was concentrated *in vacuo*, made alkaline with baryt, and extracted three times with ether. The ether solution was washed with water and the solvent was removed. Then the resulting residue was heated in a boiling water-bath with 10 ml. of 50 per cent potassium hydroxide for about 4 hours, and after cooling, solid that separated was filtered off. The mother liquor was extracted with ether, washed with water, dried over sodium sulfate, and after removal of ether, a free base with a pale yellow waxy appearance was obtained (489 mg.).

Fractionation of the Ester—The above described ester fraction was recrystallized from methanol, and white crystals thus obtained weighed 0.438 g. (38.2 per cent of the total amount of ester). On repeated recrystallization from methanol this material melted at 58–59°, and was converted by saponification with potassium hydroxide to free acid, melting at 79.5–80° after recrystallization from petroleum ether. It showed no depression of melting point when mixed with an authentic sample of lignoceric acid.

From the methanolic mother liquor, 705 mg. of a pale yellow waxy material was obtained on evaporation of the solvent. This material was fractionated by distillation into the following portions:

1) b. p. 105–115°/0.02–0.05 mm., m. p. 25–26°, 199 mg.
2) b. p. 117–140°/0.007 mm., m .p. 25–27°, 81 mg.
3) residue, 415 mg.

The portions 1) and 2) were combined (280 mg.) and saponified with methanolic potassium hydroxide. Free acid thus obtained was recrystallized to colorless plates from acetone, which melted at 58–59° and showed no depression on admixutre with an authentic sample of palmitic acid.

Base Fration—The free base described above was dissolved in warm petroleum ether, and cooled. The solid that separated out was dried and acetylated with 1.2 ml. of acetic anhydride and 1.4 ml. of pyridine in an oil bath at 142–5° for 2 hours. The crystalline material obtained was recrystallized from acetone to colorless needles, melting at 100–101°. $[\alpha]_D^{21°}=-5.44°$ (10.02 mg. in 1 ml. of chloroform)

Analysis—

Calculated for triacetylsphingosine, $C_{24}H_{43}O_5N$, C 67.73, H 10.18, N 3.29
Found: C 67.47, H 9.90, N 3.36

RESULTS AND DISCUSSION

The glycolipids which were obtained from a large amount of stroma of hog erythrocytes gave no purple coloration with Bial's orcinol reagent, and also the other lipid fractions from the same stroma did not show this reaction.

Analyses of the material obtained as glycolipid were carried out at various stages of its purification (Tables I and II). From the results of these analyses, especially from the varying figures in reducing power and hexosamine contents of each fractions, the glycolipid fraction from stroma of hog erythrocytes might possibly consist of several glycolipids differing in their sugar parts.

However, almost the same figure obtained for H_{25} and H_{26} as well as for H_{261} and H_{262} revealed that the fraction H_{261} must be considerably homogeneous glycolipid.

TABLE II

Analytical Data of H_{261} in Comparison with that of Globoside and Klenk's Glycolipid

	H_{261}	Globoside (Yamakawa and Suzuki)	Glycolipid of human blood stroma (Klenk and Lauenstein)
C %	60.16–60.20	60.42–61.27	59.82
H %	9.42– 9.60	9.30– 9.71	9.95–10.01
N %	2.07– 2.31	2.28	1.80– 1.86
Sugar %	49.4 –53.8	52.9 –56.2	40.5 –40.7
Hexosamine %	12.6 –13.3	15.6 –15.9	9.9 –10.3
Acetyl %	Indefinite (2.76–8.50)	2.16– 2.82	……
$[\alpha]_D$	+21.8°	+10.3°	……
Melting point	195–200° (decomp.)	240° (decomp.)	234°

The precipitate which was obtained on hydrolysis of H_{261} with 3N hydrochloric acid was analyzed to be a mixture of fatty acids and hydrochlorides of bases, presumably sphingosine or a sphingosine-like base.

Lignoceric acid was isolated from that precipitate as a main constituent of the fatty acids of the glycolipid, and the residual fatty acids obtained in minor amounts from the mother liquor were hardly identified by distillation or recrystallization method. However, the most part of this difficulty was overcome by means of the reversed phase column chromatography, by which almost all fatty acid components of the glycolipid were separated. The results obtained will be described in next paper (*14*).

On hydrolysis of H_{261}, there found reducing substances in solution. These were neutral sugar and hexosamine hydrochloride. It was found by paper chromatography that neutral sugar was a mixture of galactose and glucose (Fig. 1). The relatively larger spot for galactose than that for glucose obtained on the paper chromatogram showed probably a larger content of the former in the hydrolysate. Galactose was also identified as α-methyl-phenylhydrazone.

By the paper chromatographic analysis for the hexosamine components of the glycolipid, using collidine saturated with water, only chondrosamine was detected. On the other hand, in order to demonstrate the minor content of hexosamine, this method seemed to be unsatisfactory, because, according to Gardell (*13*), recovery of hexosamine on paper chromatography with such a basic solvent was reported to be less than 30 per cent. However, the cation exchanger chromatography had succeeded in their separation and determination (*13*). Using this technique, however, no glucosamine peak could be detected (Fig. 3). Hexosamine contained in the total lipid of the hog erythrocytes stroma was also examined by the same method, and was found also to contain only chondrosamine (*14*). Therefore, no glucosamine should be contained in the glycolipid of hog stroma.

The acetyl determination of H_{261} gave no values consistent enough to establish definitely its composition according to the reagents and duration of decomposition. The reason for these unusual results is yet unknown.

Sphingomyelin of hog blood stroma was also purified and identified. On hydrolysis, palmitic and lignoceric acid were identified. Further detailed analyses of the acid fraction by the reversed phase column chromatography will be described in the next paper (*14*).

SUMMARY

1. A chondrosamine-containing glycolipid was isolated from

lyophilized stroma of hog erythrocytes.

2. This is a peculiar glycolipid composed solely of chondrosamine as hexosamine component. Both galactose and glucose was found in the glycolipid, and lignoceric acid was also identified as its main fatty acid component.

3. Sphingomyelin was also isolated from stroma of hog erythrocytes, and was found to contain palmitic and lignoceric acid as the fatty acid component.

The author wishes to express his gratitude to Prof. S. Akiya and Dr. T. Ukita for encouragement. He thanks especially to Dr. T. Yamakawa for his helpful advice.

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THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

VIII. THE NATURE OF HEXOSAMINE AND FATTY ACIDS OF BLOOD CELLS SPHINGOLIPIDS

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The presence of hexosamine in lipid was first suggested by Fränkel and Kafka (*1*) early in 1920 ; they isolated " Di-lignoceryl-*N*-diglukosamin-monophosphorsäureester " from brain but its real existence is now doubtful.

In 1938, Blix claimed the occurrence of hexosamine in brain protagon fraction (*2*). Afterward, chondrosamine was isolated by Blix and his collaborators (*3, 4*) from ganglioside and successively confirmed by Klenk (*5*).

In 1951, the present authors isolated a glycolipid, hematoside, from equine blood stroma, which contains hemataminic acid but no hexosamine (*6*). On the other hand, Klenk and Lauenstein (*7*) demonstrated the absence of neuraminic acid (hemataminic acid) and presence of chondrosamine in human blood glycolipid paperchromatographically, which was subsequently confirmed by us (*8*).

The present authors have determined the contents of both hexosamine and hemataminic acid in the lipid extracts of various species of animal blood cells (*9*). According to the results, human, sheep, goat and hog blood cells glycolipid contain only hexosamine and no hemataminic acid, whereas equine and dog erythrocytes lipids possess hemataminic acid but scarcely hexosamine. In bovine and rabbit, both are present.

Shortly afterward, Klenk and Lauenstein isolated a glycolipid from bovine erythrocytes and found the component hexosamine to be glucosamine instead of chondrosamine by paperchromatography (*10*).

Recently, Gardell (*11*) has reported a method of separation of a small amount of glucosamine and chondrosamine by ion-exchange chromatography.

As shown in the precedent papers (*12, 13*), using this simple technique, only chondrosamine was found in hog blood glycolipid and the presence of small but distinct amount of glucosamine besides chondrosamine was demonstrated in the human blood cells glycolipid, or globoside, in spite of the results with paperchromatography by Klenk and Lauenstein and by us (*7*, *8*, *10*).

In this communication, similar examinations were carried out with the hydrolysate of lipid extracts of blood stroma sample of various animals. As the results, there proved to be an intimate correlation between the nature of hexosamines and animal species; namely, lipid-glucosamine and -chondrosamine of sheep and goat red blood cells exist in certain proportions, rabbit contains only glucosamine just reversely to hog's case and, in stroma of bovine, a small amount of chondrosamine was demonstrated, despite the paperchromatographic data of Klenk and Lauenstein (*10*). Such a characteristic feature of glycolipid-hexosamine contents is of special interest as compared with the results about brain ganglioside and blood plasma. The component hexosamine of ganglioside is shown to be entirely chondrosamine both in human and bovine brain and, as reported previously, the mucoprotein-constituting hexosamine of various animal sera were only glucosamine. (*14*).

As for the fatty acid component, on the other hand, the present authors had reported in the first paper of this series the component fatty acid of equine blood cells sphingomyelin is exclusively palmitic acid (*6*). Since fatty acid of sphingomyelin so far isolated by the precedent workers are in all cases a mixture of several saturated and unsaturated ones, the publication at that time was so cautious that duplicate experiments were carried out with essentially the same results that the hydrolysate contained mainly palmitic acid and a trace of lignoceric acid. However, two years later, Klenk and Lauenstein (*15*) reported in their paper concerning equine blood cells sphingolipids, lignoceric acid content is at least 30 per cent in sphingomyelin. In this report, purified equine stroma sphingomyelin was hydrolysed and the nature and amount of fatty acids are examined with reversed phase column chromatography devised by Silk and Hahn (*16*).

As a result, palmitic acid proved to be the main component if not exclusively, and lignoceric acid content is not so high as Klenk and Lauenstein (*15*) have been claimed.

Furthermore in this paper, the fatty acid chromatogram of other blood cells sphingolipids, namely, human, equine and hog erythrocytes sphingomyelin and glycolipids are also reported *en bloc*.

EXPERIMENTAL AND RESULTS

Determination of Lipid-Hexosamine—Weighed sample (200–500 mg.) of lyophilised blood stroma was extracted for 3 hours with a mixture of chloroform-methanol (1:1), after which the solvent was evaporated to dryness and the residue was heated with 2 ml. of 2 *N* HCl for 5 hours.

Any insoluble material was removed by filtration, the solution was decolorised with charcoal, concentrated under reduced pressure to dryness and was placed in an exsiccator over potassium hydroxide for a day. The residue was taken up in 0.5 ml. of 0.3 *N* HCl and chromatographed through a 0.6×40 cm. of Dowex 50 column (about 100 mesh) prepared exactly identically with Gardell's procedure. Elution was carried out with 0.3 *N* HCl at a flow rate of about 1.5 ml. per hour. Exactly 1 ml. of effluent was taken with an automatic fraction collector. The determination of hexosamine was carried out by the modified Elson-Morgan's method as described by Gardell (*11*).

The results are shown in Fig. 1. As seen in this figure, rabbits' red cells glycolipid-hexosamine is entirely glucosamine just reversely to hog's case ; bovine one contains only a small amount of chondrosamine besides glucosamine (C:G=0.23). The ratio of chondrosamine to glucosamine is 1.3 and 2.3 in sheep and goat stroma, respectively. On the other hand, the component hexosamine of human* and bovine brain ganglioside is, in each case, exclusively chondrosamine.

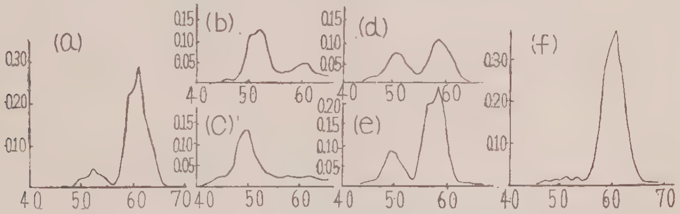

Fig. 1. Chromatographic separation of hexosamine in blood stroma of various animals. ordinate: extinction, abscissa: effluent volume (ml.)
(a) globoside, irrespective of blood group, (b) bovine, 250 mg., C:G=0.23, (c) rabbit, 500 mg., (d) sheep, 200 mg., C:G=1.3, (e) goat, 200 mg., C:G=2.3, (f) hog, 100 mg. The former peak: glucosamine, the latter peak: chondrosamine.

Reversed-Phase Column Chromatography of Fatty Acids

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* A sample of human brain ganglioside was kindly supplied by Dr. T. Nakayama of Prof. Yasuda's laboratory.

The procedure used in this study generally followed the method of Silk and Hahn (*16*).

A standard column (0.8 cm. × 85 cm.), packed with non-wetting kieselguhr impregnated with liquid paraffin, and aqueous acetone-medicinal paraffin system was used throughout. Each column was used repeatedly until an ageing effect was ap parent. The column was maintained at 35° during experiment.

PREPARATION OF MATERIALS

Non-Wetting Kieselguhr—' Hyflo Super Cel ' (John's Manville) was dried at 110° after removal of the finer particles by suspending the powder in water. The material was exposed to the vapour of dimethyl-dichlorosilane for several hours in a partially evacuated desiccator, or suspended in a solution of the silane in anhydrous ether and then evaporated. The material thus treated was washed free of hydrochloric acid with methanol and dried at 110°. The product thus obtained was completely non-wetting when shaken with water.

Medicinal Paraffin—Colorless liquid paraffin was dissolved in pure petroleum ether and passed through a column of Brockman's alumina. Evaporation of solvent gave pure paraffin and was used as stationary phase.

Acids—Approximately 0.01 m-*M.* of pure, normal saturated fatty acids with 8 to 24 carbon atoms except 12 and 20, together with oleic and nervonic acid were used as the standards. Odd numbered acids were not examined.

Acid mixtures as components of various lipids used in this study were prepared by hydrolysis of approximately 100 mg. of lipid with 3 *N* HCl, and extraction of the reaction mixture with ether. The ethereal solution was washed with water, dried over sodium sulfate, divided into two portions and after evaporation of the solvent the residue was dried in an evacuated desiccator over potassium hydroxide. Each portion was weighed. The one portion was served as natural and another portion of acid mixture was hydrogenated in ethanol with platinum oxide as catalyst. The reduced fatty acid mixture thus obtained was also chromatographed in the same manner as described with the natural acid mixture.

Developing solvent—Commercial acetone was purified as usual to prepare developing solvents. 40, 50, 70, 75, 80, 83 and 90 per cent aqueous acetone were prepared and equilibrated with liquid paraffin before use, then clarified by removing suspended oil droplets by passing through a 2–3 cm. depth of bed of kieselguhr-paraffin powder.

Procedure

Preparation of Column—In order to prepare air-free column, special precautions were taken during packing. 20 g. of the non-wetting kieselguhr impregnated with liquid paraffin was placed in a porcelain mill, together with 83 per cent aqueous acetone (200 ml) previously equilibrated with paraffin. After thorough agitation, the slurry was transferred into a 500 ml. flask and boiled to expel air. The column was filled with the boiled 83 per cent aqueous acetone, then the slurry was transferred to the column by a ' Komagome ' pipette carefully so as not to introduce air bubbles into it and allowed to fall by setting it to flow. Satisfactory air-free column obtained by this method was then eluted out with 40 ml. of 40 per cent aqueous acetone before loading the acids to be chromatographed.

Loading of Column—Following procedure was employed for loading the mixture of acids to be examined. To the acids mixture dissolved in anhydrous ether was added appropriate amount of non-wetting kieselguhr impregnated with liquid paraffin (0.3 g. for each 7 mg. of acids), and after evaporation of the solvent, the powder was placed in an evacuated desiccator to remove trace of the solvent. For loading, the powder was thoroughly slurried with 40 per cent aqueous acetone (about 5 ml.) previously equilibrated with paraffin. The mixture was boiled to expel air and poured on to the column gently by a ' Komagome ' pipette, then a small protecting band of acid-free powder was placed on top of the column. For this purpose, a small quantity (about 1 g.) of powder was boiled with 40 per cent aqueous acetone and poured on to the column.

Elution and Titration—For the chromatography of a mixture of fatty acids from C_8 to C_{24}, development was begun with 40 ml. of 40 per cent aqueous acetone. After 40 ml. eluted out, 84 ml. of 50 per cent, 68 ml. of 70 per cent, 40 ml. of 75 per cent, 34 ml. of 80 per cent, 34 ml. of 83 per cent, and 100 ml. of 90 per cent aqueous acetone previously equilibrated with paraffin were successively added from a dropping funnel fixed on the top of the column. A flow rate was from 35 ml. to 50 ml. per an hour. Successive 4 ml. portions of eluate were collected with an automatic fraction collector and were titrated with methanolic 0.01 *N* NaOH. Each tubes was boiled before titration, and bromthymol blue, as 0.1 per cent neutral solution in 70 per cent aqueous acetone, was used as an indicator.

RESULTS

A typical curve demonstrating of a mixture of seven normal sturated and two normal unsaturated fatty acids are shown in Fig. 2.

The results of chromatographing component fatty acids of human, equine and hog sphingomyelin and glycolipids are given in Fig. 3 to 8. The nature and amounts of fatty acids revealed by these Figures are essentially in agreement with the data by isolation method, already reported (*6*, *8*, *13*).

However, the quantitative relationship is much more easily shown. As seen in these Figures, the first small peak of fatty acid present in every glycolipid sample is absent in the pattern of sphingomyelin. The nature of this lower fatty acids will probably be clarified soon.

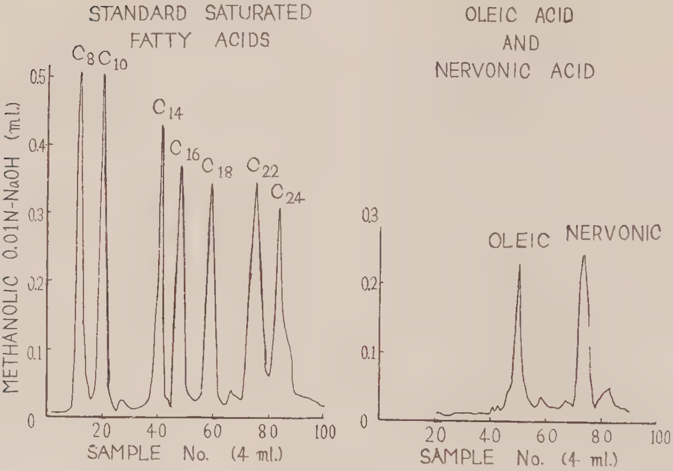

Fig. 2. Reversed-phase chromatogram of standard fatty acids.

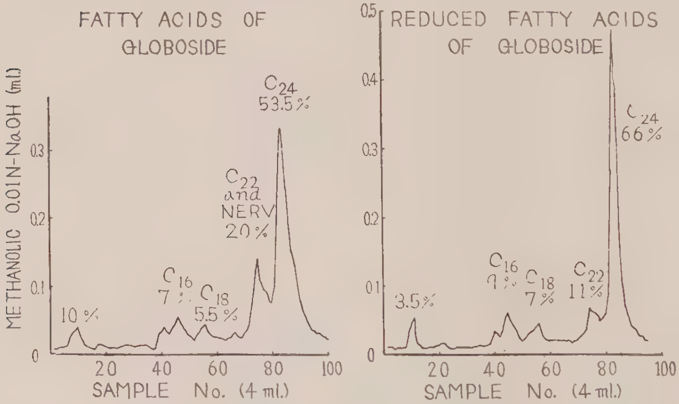

Fig. 3. Fatty acids chromatogram of human blood stroma glycolipid.

DISCUSSION

A number of studies concerning erythrocytes components have been reported in view of their serological specificity besides physiological significance. However, the detailed chemical analyses so far carried out have not explain the serological differences of animal species. For

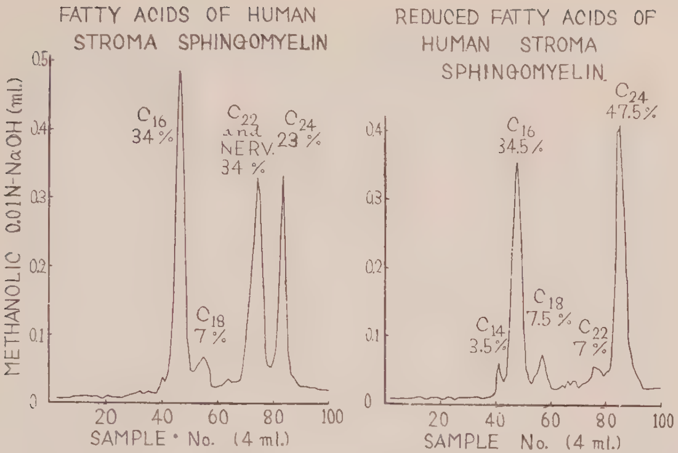

FIG. 4. Fatty acids chromatogram of human blood stroma sphingomyelin.

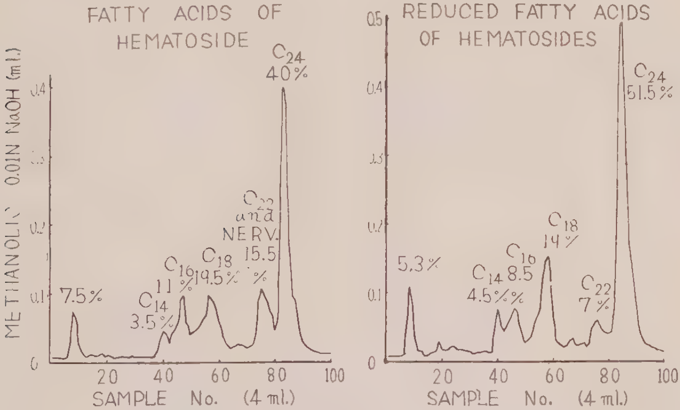

FIG. 5. Fatty acids chromatogram of equine blood stroma glycolipid.

instance, from the results of Williams, Erickson and Macy (*17*, 18) about the quantitative determination of several mammalian erythrocytes stroma with special regard to their lipid content and amino acid constitution, no remarkable differences comes out.

Recently, the studies from this and Prof. Klenk's laboratories have revealed the red cells glycolipids of several animals are chemically not

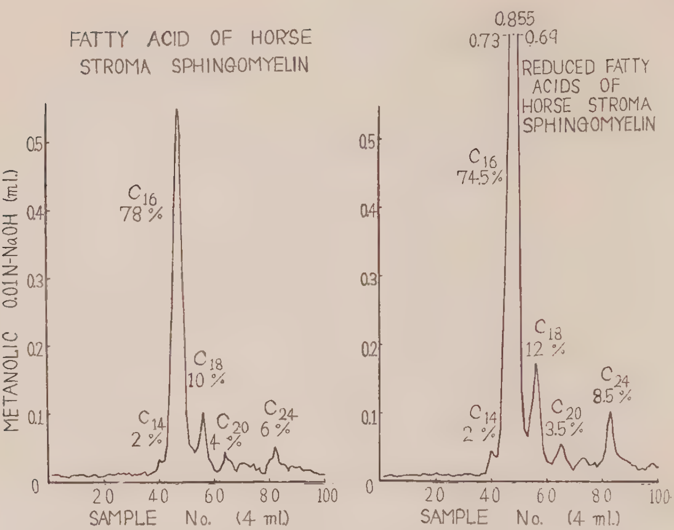

FIG. 6. Fatty acid chromatogram of equine blood stroma sphingomyelin.

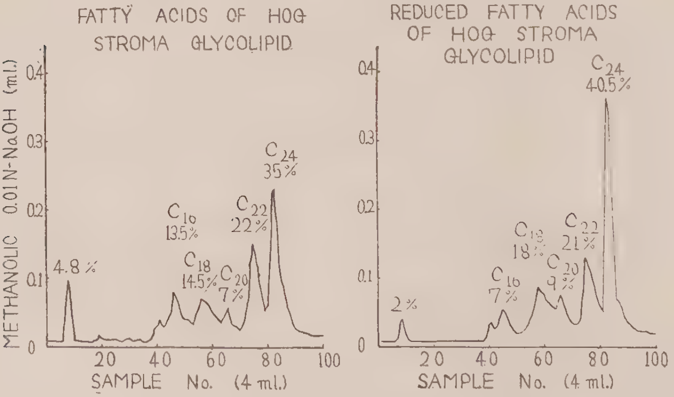

FIG. 7. Fatty acids chromatogram of hog blood stroma glycolipid.

identical with each other, though very similar. As shown in this paper, the chromatographic patterns of component hexosamines and fatty acids of red cells sphingolipids are very peculiar with respect to the animal species.

Calling in mind that the lipids of red blood cells are bound in the

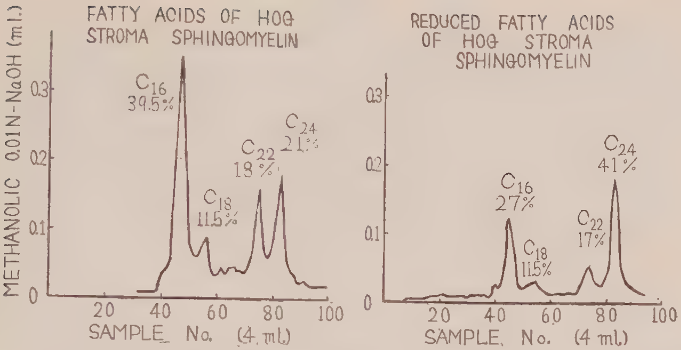

FIG. 8. Fatty acids chromatogram of hog blood stroma sphingomyelin.

framework of the erythrocytes, such a chemical peculiarity might be in some way associated with at least one of the species-specific constructions of animal body.

SUMMARY

1. By the cation exchange chromatography, the component hexosamines of several animal blood cells glycolipids were estimated separably.

2. The fatty acids which comprise stroma sphingomyelin and glycolipids were also determined by reversed phase chromatography.

3. The chromatographic patterns of them are in any instance extremely characteristic with animal species.

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KYNURENINE IN THE WINGS OF THE PAPILIONID BUTTERFLIES

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It is well known (*1*, *2*, *3*) that various pteroid pigments are found in the fluorescent substances in the extracts of wings of the Pierid butterflies when examined by means of paper chromatography. As for other families, however, little work has been done. One of the authors investigated the fluorescent substances in the wings of twenty-five species of butterflies (including *Papilionidæ*, *Pieridæ*, *Satyridæ*, *Nymphalidæ*, *Lycaenidæ*, and *Hesperiidæ*) by means of paper chromatography, and found that a white-blue fluorescent substance, which was assumed to be kynurenine, was present only in the Papilionid butterflies (*4*). This white-blue fluorescent substance has been compared with synthetic kynurenine in various points, as well as subjected to the biological test of kynurenine, and the identity of the substance with kynurenine has been proved. In addition, it has been confirmed that the substance is stored in the white-yellow or yellow scales of the wings, and that the quantity differs with the sex. The present paper gives the qualitative and quantitative data on kynurenine in the wings.

MATERIALS

The butterflies examined were the following four species:

Papilio machaon—The wings have a characteristic deep yellow and black patterns.

P. xuthus—The wings have a characteristic white-yellow and black patterns.

P. helenus—In both males and females, the fore-wings are black and do not have any white-yellow part at all, while each of the hind-wings has a large white-yellow marking on a black ground.

P. protenor—In females, both the fore-wings and the hind-wings are wholly black. In males, each of the hind-wings has a white-yellow crescent marking on a black ground, while the fore-wings are wholly black.

Some *P. xuthus* were collected in a field (Ishikawa Prefecture) and others were bred in the laboratory. As for the other three species, only those collected in the field were used. In all the experiments, the wings were used in as fresh a state as possible.

METHODS

Extraction—The wings (or parts of wings) of butterflies were cut, placed in an evaporating dish with water, and heated on the boiling water bath. The white-yellow pigments of the scales dissolved easily in hot water. The solution was centrifuged to remove the scales, and the supernatant fluid was examined. This fluid is, hereafter, called ‘the hot-water extract.’

Paper Chromatography—The hot-water extract of the wings was applied in small quantities to a marked spot on a strip of filter paper (Toyo Roshi No. 50, 1.5 × 40 cm.) and subjected to one-dimensional chromatography with either of the following solvents:

(i) B.A.W.—*n*-butanol:acetic acid:water (4:1:5)
(ii) M.B.B.W.—methanol:benzene:*n*-butanol:water (2:1:1:1).

After being developed and dried, the chromatogram was inspected under ultraviolet rays, and the area of fluorescent spots was marked with a pencil.

Color Reactions—The Ôtani and Honda's kynurenine reaction (using *p*-dimethylaminobenzaldehyde) (*5*) was applied to the hot-water extract before subjecting it to chromatography. After chromatographing, the following color tests were made on the paper:

(i) Ninhydrin reaction
(ii) Ehrlich's aldehyde reaction
(iii) Tsuda's reagent (β-diethylaminoethyl-α-naphthylamine oxalate) (*6*)
(iv) Ehrlich's diazo reaction

Ultraviolet Absorption—The hot-water extract of the wings of ten males of *P. xuthus* was spotted along the starting line 3 cm. from the margin of a sheet of filter paper (24 × 25 cm.). After being developed with the solvent B.A.W. one-dimensionally, the chromatogram was inspected under ultraviolet rays. The area of the white-blue fluorescent substance was marked with a pencil, cut out, and eluted with 1/15 *M* phosphate buffer (pH 7.35). The ultraviolet absorption of this eluate was measured between 230 and 380 mμ using a Beckman spectrophotometer (model DU).

Paper chromatography, various color tests, and ultraviolet absorption measurement were also made on synthetic kynurenine for comparison.

“*v bw and cn bw Tests*” *of Drosophila melanogaster*—Culture medium: Agar, 0.15 g.: glucose, 0.40 g.; water, ca. 3.0 ml.; phosphate buffer (pH 5.9), 0.6 ml.; the hot-water extract (or eluate), *ca.* 3.0 ml. After cooking and bottling, the medium was seeded with yeast. The double mutant *v bw* or *cn bw* of *D. melanogaster* was fed on this medium and the change of eye-color was observed.

Estimation of Kynurenine—In *P. machaon* and *P. xuthus*, the wings of one butterfly were extracted three times with 5 ml. each of hot water on a water bath, while in *P. helenus* and *P. protenor*, the markings of the wings of one butterfly were cut out and extracted twice with 2.5 ml. each of hot water in the same way. By these procedures, kynurenine in the wings was completely extracted. After the extracts were combined and the total volume measured, 4 ml. of it was taken and treated with the Tsuda's reagent (Mochizuki's method) (*7*). Its optical density was determined at 550 mμ, using Shimazu's photoelectric colorimeter, DF-III. Calibration curve with synthetic kynurenine was straight in the range of 5 to 40 γ per ml. ($y=0.015x$: x=concentration of kynurenine, y=optical density).

RESULTS

Ôtani and Honda's Kynurenine Reaction—*P. machaon*, *P. xuthus*, and *P. helenus* were examined. The hot-water extracts of the wings of these butterflies were all positive to the reaction. The purple color did not migrate to the butanol layer.

Paper Chromatography—Only *P. xuthus* and *P. helenus* were used. When the hot-water extracts of the wings were chromatographed and inspected under the ultraviolet rays, three fluorescent spots were recognized as shown in Fig. 1. The white-blue fluorescent substance (spot 1) was compared with synthetic kynurenine in respect to fluorescence, Rf value, and various color tests. Both were proved to have the same fluorescence and the same Rf value, that is, 0.43 in the solvent B.A.W., and 0.41 in the solvent M.B.B.W. Furthermore, both gave the same results in color tests made on paper as shown in Table I.

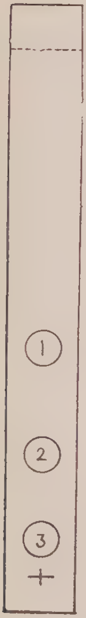

FIG. 1. Paper chromatogram of the fluorescent substances of the wings of the Papilionid butterflies. Solvent: B.A.W.

TABLE I

Color Reactions of Spot 1 of the Butterflies and Synthetic Kynurenine

Reaction	Spot 1*	Synthetic kynurenine
Ninhydrin	+	+
Ehrlich's aldehyde	+	+
Tsuda	+	+
Ehrlich's diazo	−	−

* The spot number is the same as in Fig. 1.

v bw and cn bw Tests—*P. xuthus* was examined both before and after being chromatographed. The hot-water extract of the wings was positive to the *v bw* test, but negative to the *cn bw* test. After chromatographing the hot-water extract, areas corresponding to the white-blue fluorescent substance were cut out from the chromatogram and eluted with hot water. This eluate was also positive to the *v bw* test. On the other hand, the eluate of areas containing no white-blue fluorescent substance was negative to the *v bw* test.

Ultraviolet Absorption—Only the males of *P. xuthus* were examined. The absorption curve of the white-blue fluorescent substance had two peaks, corresponding to those of synthetic kynurenine as shown in Fig. 2. In both cases, the wave lengths of the maximum absorption were 257 mμ and about 360 mμ.

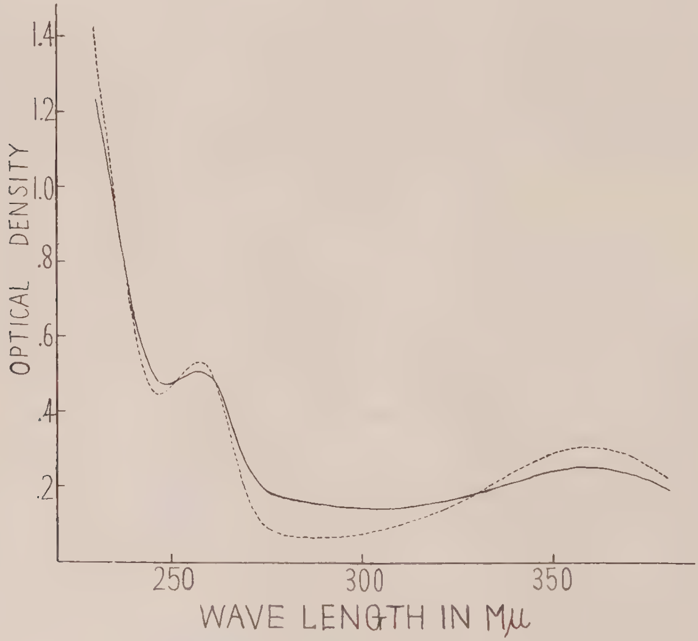

FIG. 2. Ultraviolet absorption curves of synthetic kynurenine (- - - - - -) and the white-blue fluorescent substance of the wings of *P. xuthus* (———).

From the above-mentioned qualitative tests, the white-blue fluorescent substance of the wings of Papilionid butterflies was proved to be kynurenine. Furthermore, to determine the localization the following experiments were made.

Location of Kynurenine in the Wings—*P. helenus* was used. In the wings of this butterfly, the upper side of the marking is white-yellow and the under side is white, while parts other than the marking are black. The scales of each part were separately shaved, extracted with hot water, and examined by means of paper chromatography. The results are given in Table II, where it will be seen that kynurenine is stored only in the white-yellow scales. The male of *P. protenor* was also examined to determine the location of kynurenine. The wing was separated into the white-yellow crescent marking and the black part, and each of them was separately extracted and examined by means of paper chromatography. In this case also, the presence of kynurenine was proved only in the white-yellow part.

TABLE II

Fluorescent Substances of the Scales of the Wings of P. helenus

Spot No.*	Color of fluorescence	Identity	Marking		Black parts
			Upper side	Under side	
1	White-blue	Kynurenine	+	—	—
2	Purple	Isoxanthopterin (?)	—	—	+
3	Yellowish grey	——	—	—	±

* The numbers are the same as in Fig. 1.

These results indicate that the white-yellow scales of the wings of the Papilionid butterflies accumulate kynurenine.

Estimation of Kynurenine—The wings of *P. machaon*, *P. xuthus*. *P. helenus*, and *P. protenor* were examined. In all the cases, males and females were separately examined. In every measurement, the wings of one butterfly were used. The results are shown in Table III, which gives the relative amounts of kynurenine in the wings of one butterfly. In *P. machaon* and *P. xuthus*, the males had a larger quantity of kynurenine than the females. The sex differences in these species were statistically significant, the value of P being less than 0.001. In *P. helenus*, a little more kynurenine was found in the females than in the males, but the difference was not statistically significant, the value of P being between 0.2 and 0.3. In *P. protenor*, only the males were examined, for the wings of the females had no yellow parts. Furthermore in *P. helenus* and the

TABLE III

Kynurenine in the Wings of One Butterfly

(The figures in parentheses represent the number of the butterflies examined)

	♂ γ	♀ γ	
P. machaon	276.9±21.6* (11)	162.8±16.3 (8)	$0.001 > P$
P. xuthus	320.7±24.8 (13)	192.3±19.2 (13)	$0.001 > P$
P. helenus	81.3± 6.5 (10)	97.8±10.6 (11)	$0.3 > P > 0.2$
P. protenor	32.6± 2.3 (11)	——	——

* Standard error.

males of *P. protenor*, the area of the marking was respectively measured in every butterfly examined, and the quantity of kynurenine per cm^2. of the markings of one butterfly was calculated. The mean values are shown in Table IV. In *P. helenus*, as the area of marking was a little larger in the females than in the males, the quantity of kynurenine per cm.^{2} was found to be a little greater in males than in females, although as shown above, the total quantity is greater in females. In this case also, the difference was not statistically significant, the value of P being between 0.2 and 0.1. In *P. protenor*, interestingly enough, the quantity per cm.^{2} of the markings of the males was proved to be much the same as that of the males of *P. helenus*.

TABLE IV

Kynurenine per Square Centimeter of the Markings

(The figures in parentheses represent the number of the butterflies examined)

	♂ γ	♀ γ	
P. helenus	0.33±0.02* (10)	0.29±0.07 (11)	$0.2 > P > 0.1$
P. protenor	0.32±0.02 (11)	——	——

* Standard error.

DISCUSSION

As for the fluorescent pigments of the wings of butterflies, the presence of pteroid pigments has already been reported in Pieridæ (*2*). Leucopterin and isoxanthopterin are contained in large quantities in the white wings of *Pieris rapae*, *P. melete*, and others, while xanthopterin and xanthopterin-B are present in large quantities in the yellow wings of *Colias hyale*, *Eurema hecabe*, and *Gonepterix mahaguru*. However, little work has been done on the wings of the other butterfly families. For this reason, it is interesting that the wings of the Papilionid butterflies have been proved by the present series of investigation to contain a large quantity of kynurenine. In the butterflies thus far examined by the authors, those which store kynurenine in the wings have been only Papilionidæ.

In a previous paper (4), the white-blue fluorescent substance of the wings of 4 genera 8 species (including *Parnassius*, *Lühdorfia*, *Papilio*, and *Graphium*) in Papilionidæ was examined by means of paper chromatography. From the results in both the preceding paper and the present paper, it may be definitely concluded that among the Papilionid butterflies thus far examined, those which accumulate kynurenine in the wings are only *Papilio* (*P. machaon*, *P. xuthus*, *P. helenus*, and *P. protenor*) and *Lühdorfia* (*L. japonica*), and that kynurenine is found only in very small quantities in *Parnassius glacialis*, and not at all in *Graphium sarpedon*. These results indicate that in the wings of Papilionidæ, kynurenine is stored up only in those which have a white-yellow or yellow markings. For example, even in *Papilio*, the wings of *P. bianor*, which are black and have no yellow part, have no kynurenine. This is in agreement with the results of the present tests, which indicate that in the wings of *P. helenus* and *P. protenor*, kynurenine is accumulated only in the white-yellow markings.

The above-mentioned discussion also suggests the possibility that the presence or absence of kynurenine in the wings may be useful for systematic studies on Papilionidæ. Up to the present, anthoxanthin and red pigments have already been reported as pigments in the wings of the Papilionid butterflies. Ford (*8*) referred to the presence of anthoxanthin and the types (A or B) of red pigments as a basis for examining the affinity of the Papilionid butterflies. In such an attempt, kynurenine had better be taken also into consideration in the future.

It is now necessary to consider the relation between kynurenine and the yellow pigments of the wings. On the whole, the fact that kynurenine is stored up only in the yellow parts suggests that these pigments may be closely related to kynurenine. However, the pigment seems not to be of one kind but to be of various kinds. For example, the white-yellow pigment of the wings of *P. xuthus*, *P. helenus*, and *P. protenor* are easily soluble in hot water, but the deep yellow pigment of the wings of *P. machaon* is hardly soluble in hot water. The relation between these various pigments and kynurenine must be a subject of future researches.

The reason why kynurenine is accumulated in the white-yellow markings is not clear. This is not to be discussed in the same manner as are the mutants such as *cn* of *D. melanogaster* and *white*-1 of *Bombyx mori*, which are entirely lacking in the enzyme which transofrms kynurenine to 3-hydroxykynurenine, because *P. xuthus* has 3-hydroxykynurenine and xanthurenic acid in the head and abdomen (unpublished data). However, it is interesting that the accumulation of kynurenine in the markings will be compared with genetical biochemistry of tryptophan, which is advanced in regard to *Diptera* and *Lepidoptera*.

Recently, the relations between melanin, tryptophan, pterin, uric acid, xanthine oxidase, riboflavin, and metals have become an interesting problem in connection with pigment formation of insects (*9*, *10*). The fact that kynurenine is stored up in the white-yellow marking may also be taken to indicate the existence of such relationships. The white-yellow markings of the Papilionid butterflies may possibly be formed through the integrated metabolism of the above-mentioned substances. Further experiments are being carried out on the biochemical study of the markings.

Lastly, the reason why kynurenine is more abundant in the wings of the male than in the female of *P. machaon* and *P. xuthus* is not clear. In *P. xuthus*, the total area of the wings is larger in the female than in the male, while it does not differ with the sex in *P. machaon*. At any rate, the sex difference of kynurenine in the wings is interesting. In *B. mori*, kynurenine has been known to be stored up in eggs (*9*). In *D. melanogaster*, kynurenine is also found in greater quantity in the abdomen of the female than in that of the male (unpublished data). It is interesting that among insects, sex difference in the eggs and the abdomen is opposite to that of the wings. The biochemistry of sex difference of kynurenine must be another subject of future researches.

SUMMARY

1. The white-blue fluorescent substance in the extracts of wings of the Papilionid butterflies reported in the previous paper by the

authors, was proved to be identical with kynurenine both by comparison with synthetic kynurenine and by biological test.

2. Using the markings on the wings of *P. helenus* and *P. protenor*, the substance (kynurenine) was proved to be stored up only in the white-yellow scales and not in the black scales.

3. Kynurenine in the wings of *P. machaon*. *P. xuthus*, *P. helenus*, and *P. protenor* was colorimetrically determined separately with regard to sex. In *P. machaon* and *P. xuthus*, the quantity of kynurenine was proved to be larger in the males than in the females. In *P. helenus*, sex difference was not recognized. In *P. protenor*, only the males were examined. In the latter two species, furthermore, quantity of kynurenine per $cm.^2$ of the markings was also estimated.

4. The relation of kynurenine to the pigments of the wings and the markings, and the possible systematization of the Papilionid butterflies according to kynurenine content were discussed.

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STUDIES ON PROTEIN DENATURATION BY SURFACE CHEMICAL METHOD

III. THE INTERACTION OF UREA WITH LYSOZYME MONOLAYER

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Previously, the author studied on the effect of urea on the monolayers of synthetic polypeptides, such as poly-DL-α-aminocaprylic, poly-DL-α-aminocapric, poly-DL-α-aminolauric acid and poly-γ-benzyl-L-glutamate (*1*). It was found that their molecular weights determined by surface pressure measurements on the substrates with or without urea were the same, whereas the co-area of the polypeptide molecule was increased with the urea concentrations in the substrates. This fact was explained by the binding of urea to the polypeptide backbones. In the present paper, the effect of urea on the monolayer of lysozyme was examined for the purposes of elucidation of the mechanism of urea denaturation of proteins and of the interaction of urea with proteins. Protein denaturation by urea involves many complicated problems not yet solved ; especially the interaction between proteins and urea remains obscure. In the previous two papers of this series (*2, 3*), it was repeatedly emphasized that even if the proteins with rigid structures such as lysozyme are spread at interfaces, they may continue to maintain the some parts of their organized structures. The effects of urea on the monolayers of such proteins were different from those on the synthetic polypeptide monolayers. Examinations of surface pressure—area curves of lysozyme films on the substrates containing different amounts of urea, led us to the suggestion that urea denaturation of lysozyme may proceed with a stepwise process in solutions and that urea molecules may form a complex with lysozyme. Comparative experiments were also done for the monolayers of ovalbumin and poly-DL-leucine.

EXPERIMENTAL

Materials and Methods

Crystalline lysozyme and ovalbumin were provided by Prof. S. Akabori, Mr.

K. Ohno and Mr. I. Haruna, and poly-DL-leucine by Dr. H. Tani and Mr. H. Yuki in the Faculty of Science of the Osaka University.

Lysozyme and ovalbumin were dissolved in a urea solution in concentration of 8 *M*, to which 40 per cent of isopropyl alcohol was added prior to spreading. Poly-DL-leucine was spread from its chloroform solution. Surface pressure was measured by the hanging plate method of Wilhelmy type.

In the case of lysozyme and poly-DL-leucine, the substrate was adjusted to pH 10.5 with potassium carbonate. In the case of ovalbumin the pH's of the substrates were adjusted by hydrochloric acid for lower pH values and potassium carbonate for higher pH's. The urea used was a commercial product of Toyo Koatsu Kogyo Co., which was used after repeated recrystallization and adsorption by active carbon. The concentrations of urea were determined by the measurements of density, of which data were obtained by Gucker, Gage and Moser (*4*).

Results

The variations of the surface pressure-area (F-A) curves of the films of lysozyme and of poly-DL-leucine with the concentrations of urea in the substrates at constant pH (10.5) are shown in Figs. 1 and 2. The temperatures of the substrates in the case of lysozyme and poly-DL-leucine were 19° and 25°, respectively. The films expanded considerably on the substrates containing urea as shown in Figs. 1 and 2. The effect of urea on the F-A curves of the films of lysozyme on the substrates at 29° was also examined (not shown). The temperature affects the F-A

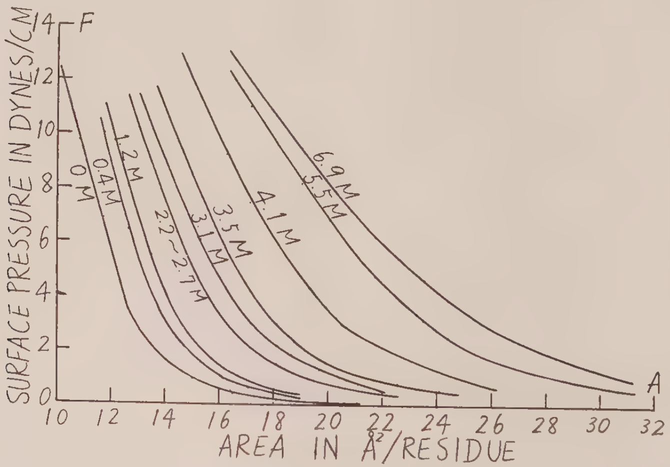

FIG. 1. The effect of urea on the F-A curves of lysozyme monolayers. The substrates: pH 10.5, 19°.

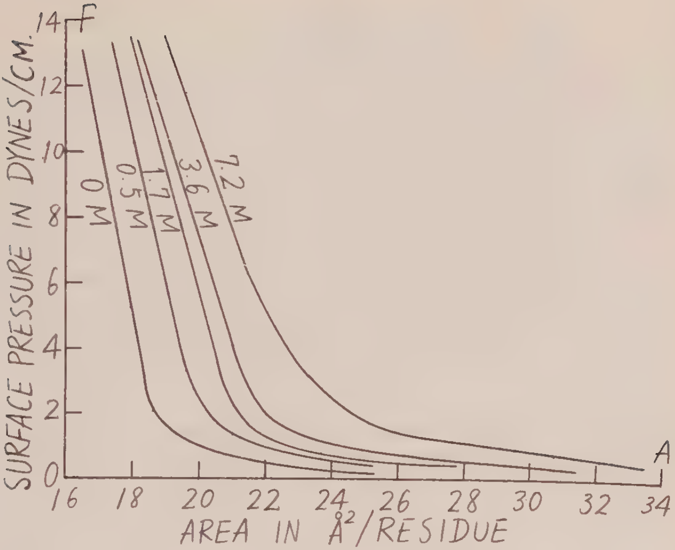

FIG. 2. The effect of urea on the F-A curves of poly-DL-leucine monolayers. The substrates: pH 10.5, 25°.

curves of lysozyme on the substrates containing urea but not that on the substrate without urea.

The F-A curves of ovalbumin on the substrates of three different pH values (pH 1.0, 4.6 and 10.5), maintaining the concentration of urea to be constant (3.1 *M*), are shown in Fig. 3. The F-A curves on the urea solutions were shifted to larger areas parallel to the curves on the substrate without urea and the effect of pH's was not observed.

If the areas at a given constant pressure on the substrates with and without urea are indicated by A and A_0, respectively, $(A-A_0)$ represents the increase in area at the pressure by the presence of urea in the substrate. In Fig. 4 the increases in area, $(A-A_0)$ at 5 dynes/cm., are plotted against the concentrations in the substrates (c) for the films of lysozyme at two different temperatures (19° and 29°) and of poly-DL-leucine (at 25°).

Three steps appeared in the $(A-A_0)$–c curve of lysozyme at 19° (the first step appeared between about 0.5 and 1.5 *M*, the second, between about 2 and 2.8 *M* and the third, above 2.8 *M*), but only two steps at 29°. (the first step appeared between 1.5 and 3.2 *M* and the second, above 3.2 *M*). In the case of 29°, the first step at 19° disappeared

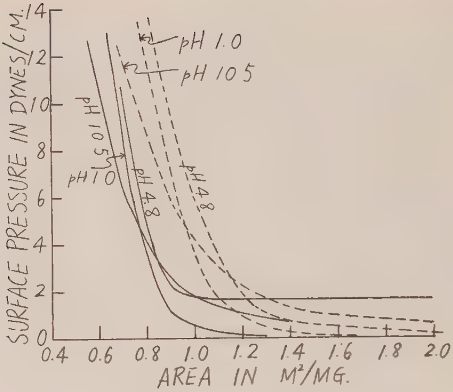

FIG. 3. The effect of pH on the F-A curves of ovalbumin monolayers on the substrates without urea (solid lines) and with urea (3.1 M) (dotted lines). The temperature of substrates: 27°.

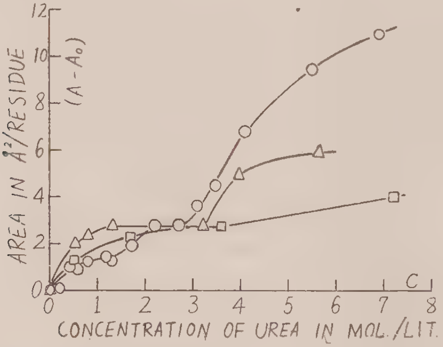

FIG. 4. The relationship between the increase in area at 5 dynes/cm. and the concentrations of urea in the substrates. ○, lysozyme at 19°.; △, lysozyme at 29°; □, poly-DL-leucine at 25°.

and the first step was found at the same height as that of the second step at 19°. In the region of lower concentrations of urea in the substrates, the expansion of the F–A curves by urea was greater at higher temperatures, but the reverse was observed in the region of higher concentrations of urea (more than about 3 M). The F–A curves did not expand on the substrate containing 1 M urea at 9°. The same was observed on the urea solution in the concentration of 0.5 M at 17°.

DISCUSSION

The effects of urea on the monolayers of cellulose acetate and synthetic polypeptides were examined by Borgin and Johnson (*5*) and by Mishuck and Eirich (*6*), respectively. They considered that secondary bonds of these high polymers which would not be broken by surface forces on distilled water should be broken by urea molecules in the substrate and that this is the cause for the increase in area with the concentrations of urea. So, it was scarcely considered that urea molecules would be adsorbed on the molecules of polypeptides or cellulose acetate. As mentioned above, however, we reported in the previous paper on the effect of urea on the films of polypeptides (*1*) that in spite of the increase of the co-area of the polypeptide molecule with the rise in the concentrations of urea, the number of molecules remained the same and the molecular weights determined by Bull's method (*7*) were the same irrespective of the urea concentrations in the substrates. This fact was explained by the binding of urea molecules to the polypeptide backbones.

In order to explain the results on lysozyme shown in Fig. 4, the following four possibilities would be suggested:

(1) Following Borgin and Johnson (*5*) and Mishuck and Eirich (*6*), the increase in area would be caused by the cleavage of secondary bonds in lysozyme molecules by urea attack which would not be broken by surface force. The breaking of bonds by urea would proceed stepwise with the increase in urea concentration.

(2) The adsorption of urea on the lysozyme molecules would increase the area. This was the conclusion obtained from our experiments on the effect of urea on the monolayers of synthetic polypeptides (*1*).

(3) (a) Urea might form a definite complex with the protein molecules (the reason for the appearance of plateaus in the $(A-A_0)-c$ curve). (b) Therefore, urea molecules might be adsorbed on lysozyme molecules by the mechanism of activated adsorption.

(4) The various polar groups in protein molecules, such as peptide bond in the backbone, carboxyl and amino groups, *etc.*, in the side chains, might be able to combine with urea molecules. However, these groups would have different affinities for the urea molecules. Therefore, a protein molecule would resemble a composite surface. The adsorption isotherm for a composite surface is characterized by a stepwise curve, details of which were reviewed by deBoer (*8*).

The F-A curves of the films of proteins have almost the same limiting area irrespective of protein species (0.85 m.^{2}/mg.) (*9*). Furthermore, synthetic polypeptides with shorter side chains which have no such an organized structure as proteins, also have the same limiting area as proteins (*10*). These facts suggest that the increase in area would not be caused by only the breaking of secondary bonds by urea attack which remain intact on the substrate without urea (possibility (1)). Therefore, the adsorption of urea on the protein molecule must be taken into account (possibility (2)). By assuming the mechanism of activated adsorption for the adsorption of urea on the protein molecules (possibility (3b)), the fact that the areas of the films became larger with the rise of temperature, might be explained. However, it cannot be explained by only the mechanism of activated adsorption that the first step in the $(A-A_0)$—c curve at 19° disappeared in the case of 29°. In order to explain this fact, it is also necessary to consider the possibility (1). That is, the higher the temperature of substrate becomes the easier are the breaking of secondary bonds of the protein molecules and the urea molecules are adsorbed on the new bonds emerged by the breaking of the intact bonds, resulting the increase of the area of the films. The appearance of the plateaus in the curves shown in Fig. 4 suggests the complex formation between urea and lysozyme molecules (possibility (3a)). The possibility (4) may be denied by the reasons described in the later part.

Summarizing the above considerations, the results shown in Fig. 4 can be explained satisfactorily as follows:

Urea molecules in the substrate break the some parts of the organized structure of lysozyme molecule which may be intact by spreading. The new groups emerged by breaking of these secondary bonds of lysozyme molecules would combine with urea molecules. The adsorption of urea on the protein molecules is the main cause for the increase in area. There are some definite weak points of structure in the molecules of the films. These weak points are broken stepwise with the increase in urea concentration in the substrate. These considerations are supported by the fact that there appeared two steps in the $(A-A_0)$–c curve at 19° and that the first step in the curve at 29° coincided with the second step at 19°. There are no sites of combination with urea molecules without breaking of the secondary bonds. This is explained by the fact that the F-A curves did not expand on the urea solutions in the concentrations of 1 or 0.5 *M* at the temperatures of 9° or 17°. It is the suggestion of a definite complex between urea and lysozyme molecules that there existed two plateaus at 19° and one at 29°. The facts that the first step at 19° disappeared at 29° and that the first step at 29° coincided with the second step at 19°, suggest that the breaking of bonds proceeds to the second step by the presence of small amounts of urea owing to higher temperature of the substrate. In this case the mechanism of activated adsorption may also be taken into account, but it cannot be decided by the present experiments.

When the concentrations of urea were greater than about 3 *M*, the areas of the lysozyme films increased much greatly. However, the film of poly-DL-leucine was not so markedly expanded in this region. Furthermore, the effect of temperature on the expansion of F–A curves in this region was reverse to that in the region of lower urea concentrations. These facts might be illustrated by either or both the following reasons: (1) That involved in greater disorganization of lysozyme molecules and (b) that involved in the multimolecular and non-specific adsorption of urea by lysozyme molecules.

From the above considerations, it is suggested that lysozyme molecules may be denatured slightly in the urea solution in the concentration of 8 *M* and that they may continue to maintain some parts of organized structures even if spread at interfaces, differing from the usual protein monolayer such as egg albumin.

Next problem is to examine that which groups of lysozyme molecule play a role of combining with urea molecules and that with what ratio of urea and lysozyme molecules the complex is formed. As mentioned above, when the structure of lysozyme remains compact, there may be no sites which can combine with urea. As the increase in area is used in the present experiments as a measure of the interaction between lysozyme and urea molecules, the adsorption of urea which does not contribute to the increase in area was out of our discussion. The urea molecules which combine with polar groups existed at the air-water interface have a greater contribution to the increase in area, but those combined with polar groups existing below the interface have a little or no contribution to the expansion of the film. Therefore, from the present experiments, it cannot be determined with what groups urea forms the complex. A suggestion for this problem, however, will be presented in the later part of this paper.

These circumstances can be well understood from the experiments on the effect of urea on the ovalbumin films (Fig. 3). When the pH's of the substrate were changed at constant concentration of urea (3.1 *M*) the shapes of the F–A curves were the same as those on the substrate without urea. If the urea adsorbed on the different kinds of ionizable groups of side chains contribute to the increase in area, the F–A curves might be affected by the pH's of the substrate and the shapes would be different from those on the substrate without urea. The F–A curves were of expanded type on the substrate at pH 10.5 and did not depend on whether the substrate contained urea or not. This fact indicates that urea would not affect the ionization of carboxyl or other groups which are ionizable at alkaline pH's. From these considerations possibility (4) would be highly improbable.

From the results on the effect of urea on the monolayer of poly-DL-leucine the conclusive evidence for the complex formation between the polypeptide and urea molecules was not obtained. However, the increase in area of poly-DL-leucine by the presence of urea was nearly the same as that of lysozyme. In the case of poly-DL-leucine it is only the peptide bonds in the backbone that involve the binding for urea molecules. Accordingly, in the case of lysozyme, the increase in area might be caused mainly by the binding of urea molecules with peptide bonds in the backbone. The binding of urea with peptide bonds in the backbone was suggested by Haurowitz. (*11*)

The effect of urea on the expansion of the films decreases in the order: poly-DL-α-aminolauric acid$>$poly-DL-α-aminocapric acid$>$poly-DL-α-aminocaprylic acid, poly-DL-leucine (see Figs., 1, 2, 3 and 4 in reference(1)). That is, the longer become the hydrocarbon side chains the more marked was the effect of urea on the expansion of the films. The films of poly-DL-α-aminolauric or poly-DL-α-aminocapric acid consist of the molecules having very compact structure on the distilled water because of the strong intra-molecular van der Waals forces between their longer hydrocarbon side chains. However, this is not the case for the films of poly-DL-leucine or poly-DL-α-aminocaprylic acid having shorter side chains. When the molecules having longer side chains are cleaved by urea these longer side chains rather contribute to the expansion of the films. So, the increase in area by urea was marked for the films of the polypeptide with longer side chains.

The facts described so far suggest that the urea denaturation of proteins in solution of constant concentration and at constant temperature might proceed at a definite step, that the binding of urea with protein molecules might be very strong and that the mechanism of urea denaturation suggested by Haurowitz (*11*) is highly reasonable.

SUMMARY

The effect of urea on the monolayers of lysozyme, ovalbumin and

poly-DL-leucine was examined by surface pressure measurements. The results obtained were as follows:

1. The areas of the films of lysozyme were increased stepwise with the increase in the concentrations of urea in the substrate. In the relation between the increases in area and the concentrations of urea, two steps appeared at 19° and one at 29° in the concentration range below about 3 *M*. The latter step appeared at the same height as the second step at 29°. The increases in area became larger with the rise of temperature of substrate in this concentration range, but the reverse was observed above this range.

2. There appeared no distinct step in the case of poly-DL-leucine. However, almost the same increases in area as for lysozyme were found on the urea solutions of lower concentrations. It was the striking difference that with poly-DL-leucine such a greater increase in area as for lysozyme was not observed in the range of higher urea concentrations.

3. The effect of pH on the expansion of F–A curves of ovalbumin films on the substrate of constant urea concentration was examined. The F–A curves were shifted to larger areas parallel to those on the substrates without urea. The specific effect of pH on the expansion was not observed.

4. The facts described above were explained by the cleavage of bonds by urea attack which remain intact by spreading and by the adsorption of urea on the bonds newly emerged by the cleavage. The binding of urea with the protein molecules was so strong that leads to the suggestion of complex formation. Urea may not affect the ionization of carboxyl, amino and the other ionizable groups. The urea bound to the peptide groups in the backbone mainly contributes to the increase in area. The mechanism of urea denaturation suggested by Haurowitz seems to be adequate.

The author wishes to express his sincere thanks to Prof. T. Isemura for his kind guidance throughout the present work and to Prof. S. Akabori, Mr. K. Ohno, Mr. I. Haruna, Dr. H. Tani and Mr. H. Yuki in the Faculty of Science of the Osaka University who kindly supplied the valuable samples of lysozyme, ovalbumin and poly-DL-leucine to him.

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PREPARATION OF FLAVIN ADENINE DINUCLEOTIDE FROM EREMOTHECIUM ASHBYII

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Flavin adenine dinucleotide (FAD), the prosthetic group for many yellow enzymes, was first prepared by Warburg and Christian (*1*) from animal tissues and yeast. This procedure has been modified and revised by other workers who made use of a chromatopile (*2*), various kinds of adsorption and partition chromatographies (*3*), chromatography on the powdered cellulose (*4*), anion exchange resins (*5*), or electrophoresis on a powdered cellulose column (*6*). However, in all these cases the starting materials for FAD were restricted to animal tissues or yeast.

Recently, Kuwata and others (*7*) reported that FAD was formed in the mycelium of *Eremothecium ashbyii* under optimal culture conditions, and that relatively pure FAD could be obtained from this mycelium.

The simplified method of preparing FAD from the fungi using circular paper chromatography was presented previously as a short report by the senior authors (*8*), and this method is suitable for the preparation of a relatively small amount of FAD (20–30 mg.) in a laboratory.

Many procedures effective for the purification of FAD from *Eremothecium ashbyii* were examined, and a relatively simple method applicable to preparation of a considerable amount of FAD in a laboratory was devised. The standard procedure is described in this paper.

EXPERIMENTAL

Analytical Method

For the separatory determination of flavin compounds, the paper electrophoresis (*9*) and spectrophotometry were used. Riboflavin nucleotides moved with different mobilities on a buffer-moistened filter paper by the effect of electric current. The separated flavin compounds were eluted with water, and the flavin concentration of the eluate was determined by measuring the light absorption at 450 mμ using the Beckman DU spectrophotometer.

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In the latter half of the procedure, the content and purity of FAD were estimated by the direct measurement of absorption at 450 mμ and 260 mμ. As the molecular extinction coefficient of FAD at 450 mμ, the value 11.3×10^3 *lit.* $mole^{-1}$ $cm.^{-1}$ was used as reported by the earlier workers (*1*, *4*). The ratio of light absorption at 260 mμ and 450 mμ was used as a criterion of purity.

Cultivation of Eremothecium ashbyii

Culture Medium and Condition of Culture—The medium was of the following composition: 0.5 g. of peptone, 1 g. of glucose, 0.2 g. of KH_2PO_4, 0.1 g. of $MgSO_4 \cdot 7H_2O$, and 0.1 g. of NaCl, made up to 100 ml. with water; pH adjusted to 6.0. For the cultivation, the flask containing inoculated medium was shaken at the frequency of 120 per minute at 30°.

Content of FAD in the Mycelium—The quantity of flavins in the mycelium reached the maximum on the third day as shown in Table I. During the first 3 days, the mycelium increased in mass, but after this period it formed spores which contained a small amount of flavin. Therefore, the mycelium must be harvested after 3 days for the preparation of FAD.

Extraction and Purification of FAD

Extraction of Flavins with Warm Water from the Mycelium—The senior author (*10*) reported that FAD could be extracted from animal tissues with warm water in a higher yield than with any other organic solvent. Similar extraction method used in the present case was as follows: 60 g. of wet mycelium, containing 102 mg. of FAD and separated by centrifugation from the medium, was placed in 200 ml. of distilled water and warmed at 75° for 15 minutes with agitation. The aqueous extract was separated by centrifugation at 3,000 r.p.m. for 15 minutes and the residue was extracted twice more with warm water as described above.

Thus, 570 ml. of combined extract obtained, gave 96.4 per cent yield of FAD. The yield of FAD and riboflavin in each extraction step

TABLE I

Biosynthesis of Flavins by Eremothecium ashbyii in Shaking Culture

Shaking frequency, 120 per minute ; Temperature, 30° ; Medium : 0.5% peptone, 1.0% glucose, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% NaCl (pH 6.0).

days			2	3	4	6	8	10
Total riboflavin	medium (*γ/ml.*)		13.1	31.2	50.2	73.0	75.0	78.5
	mycelium (*γ/mg.*)		7.8	10.3	6.8	2.3	2.4	2.4
Ratio of flavins (%)	medium	FAD	trace	15	28	36	38	40
		FR	100	85	72	64	62	60
	mycelium	FAD	46	51	61	72	—	—
		FR	54	49	39	28	—	—
Content of FAD in dried mycelium (*γ/mg.*)			4.6	6.8	6.0	2.6	—	—
Weight of dried mycelium (*mg./100 ml.*)			28	46	44	42	42	42

FR : Free Riboflavin.

TABLE II

Extraction of Flavins with Warm Water from the Mycelium

Number of extraction with 2 volumes of water at 75°, for 15 min.	Yield of flavins			
	FAD		ER	
	mg.	%	*mg.*	%
Wet mycelium (60 g.)	102.0	——	98.0	——
1	64.2	62.9	53.7	54.8
2	26.9	26.4	28.9	29.4
3	7.2	7.1	9.4	9.6
Total	98.3	96.4	91.0	93.8

is summarized in Table II.

The crude aqueous extract was stable when frozen but not at room temperature.

Concentration of Aqueous Extract and Extraction with Phenol—The aqueous extract was concentrated under a reduced pressure at 30° to a syrup. The dark brown syrup was poured into 1 *l.* of dehydrated alcohol. The yellow orange flocculent precipitate thus obtained was separated by filtration through a Bucher funnel and dried *in vacuo*. The alcoholic filtrate was yellow by the presence of riboflavin. Thus, in this procedure a large amount of riboflavin was removed and FAD was coprecipitated with denaturated protein.

The dried precipitate was extracted with four 25 ml. portions of liquid phenol, until the phenol-insoluble residue turned ash-white. Two volumes of ether were added to the phenolic solution and the mixture was extracted with four 25 ml. portions of water. The aqueous extract was washed twice with equal volume of ether.

When this aqueous solution was examined by paper chromatography using *n*-butanol-acetic acid-water mixture (4 :1 :5, *v.*/*v.*) as the developing solvent, yellow fluorescent spots of FAD and riboflavin, and blue fluorescent spot (Rf=0.14) were detected under ultraviolet light.

In this step, 91.2 mg. of FAD and 37.0 mg. of riboflavin were obtained, and the dry weight purity of FAD was 2.8 per cent.

Application of Counter-Current Distribution Method—The aqueous extract of mixed flavins was subjected to a counter-current distribution, water and water saturated benzylalcohol being used as two solvents. After six transfers, 92.7 per cent of FAD was found in the first tube and 7.3 per cent in the second tube. A portion of blue fluorescent substance migrated to the second and third tubes, but the majority of it remained in the first tube. The aqueous layers of the first and second tube were combined and this portion contained no riboflavin but only the blue fluorescent substance as described above.

In this step, 76.2 mg. of FAD, was obtained, having a purity of 7.6 per cent. The absorption ratio E_{260}/E_{450} was 9.0.

Adsorption Chromatography on "Florisil"—The aqueous fraction of crude FAD was passed through a column (6 cm. in diameter) filled with 200 g. of "Florisil" (60–100 mesh) to a height of about 27.0 cm. FAD was adsorbed at the top of the column. After the aqueous solution was passed through, the column was washed consecutively with 4 *l.* of 2 per cent acetic acid, 4 *l.* of distilled water, and about 1 *l.* of 0.5 per cent pyridine as described by Dimant and others (*3*). Thus, the pterin-like, blue fluorescent substance and coexisting nucleic acids were completely eluted with 2 per cent acetic acid and with water. By washing with 0.5 per cent pyridine, the adsorbed band of FAD was moved to the bottom of the column. Then, FAD was eluted with 5 per cent pyridine. Since FAD was shown to be converted to the Fourth Flavin Compound (FFC) (*11*) by prolonged adsorption on " Florisil ", the procedure must be operated as rapidly as possible (within 2–3 hours). Bessey and others (*12*) reported that FAD was converted to FMN by adsorption on " Florisil " at neutral pH, but in these experiments no such conversion to FMN was recognized.

The solution of FAD was washed with equal volume of chloroform five times and filtered through a filter paper previously moistened with water.

The orange yellow filtrate gave spot of neither blue fluorescent substance nor riboflavin on paper chromatogram.

The yield of FAD was 71.3 mg. Absorption ratio E_{260}/E_{450}, 3.47 ; purity of dried material, 19.2 per cent.

The aqueous solution of FAD thus obtained was saturated with ammonium sulfate and extracted with liquid phenol. The yellowish brown phenol layer was added to 1/4 volume of benzyl alcohol and dried by the addition of anhydrous sodium sulfate. The dehydrated phenol-benzyl alcohol mixture was added to two volumes of ether and extracted repeatedly with a small amount of water.

After the aqueous solution of FAD was poured into five volumes of absolute alcohol, the precipitate was separated by centrifugation and dried *in vacuo*.

In this step, 103 mg. of dried yellow powder was obtained, which contained 55.0 mg. of FAD. The absorption ratio E_{260}/E_{450} of the aqueous solution was 3.34.

Further Purification of FAD—For further purification, a powdered cellulose chromatography similar to the method of Whitby (*4*) was used. The dried FAD powder was dissolved again in 50 ml. of water, adsorbed on about 5 g. of powdered cellulose (Whatman ashless) and then dried *in vacuo*. A glass chromatographic tube (6×60 cm.) was filled with tamped powdered cellulose to a height of about 40 cm. The adsorbate of FAD was placed on the top of this column, and the mixture of *n*-propanol, *n*-butanol and water (2:2:1, *v./v.*) was allowed to flow down at the rate of 18 ml./hour. After five days, FAD fraction was cut out, eluted with warm water, and filtered through a sintered glass filter (No. 4). The solution was concentrated to about 10 ml. under a reduced pressure and then lyophilized to dryness.

In this step, 47.3 mg. of dried orange powder was obtained. Absorption ratio E_{260}/E_{450}, 3.24 ; purity, 85.3 per cent.

TABLE III

Yield and Purity of FAD in Each Step of the Procedure

Procedure	Yield (as dried material) (*mg.*)	Content of FAD (*mg.*)	Purity (%)	Absorption ratio E_{260}/E_{450}
Mycelium (wet, 60 g.)	—	102	—	—
Warm water extraction	—	98.2	—	—
Concentration under reduced pressure	—	—	—	—
Precipitation with dehyd. alcohol	—	—	—	—
Phenol extraction of the precipitate	—	—	—	—
Water extraction of phenol-ether mixture	3255*	91.2	2.8	—
Counter-current extraction	1002*	76.2	7.6	9.0
Florisil adsorption chromatography	371*	71.3	19.2	3.47
Elimination of inorganic salts	103.0	55.0	53.6	3.34
Powdered cellulose chromatography	—	—	—	—
Lyophilizing	47.3	40.4	85.3	3.24

* In this procedure these samples were obtained as aqueous solution.

Examination of the Final Preparation

The absorption spectrum of the preparation is shown in Fig. 1. This preparation gave, by paper chromatography with a *n*-butanol-acetic acid-water mixture (4 :1 :5 by volume) as the developer, a single yellow fluorescent spot at Rf 0.03.

Fluorescence of the aqueous solution of this sample was yellow and the maximum of its fluorescence spectrum was at 535 mμ. This fluorescence was strengthened by hydrolysis with *N*/10 HCl, whereby FMN was found in the hydrolysate.

The sample was also examined by D-amino acid oxidase reaction mixture. The sample was added to apo-enzyme (protein E obtained from hog kidney by the method of Negelein and Brömel) and DL-alanine, and it was proved that this was an essential agent for the reaction to take

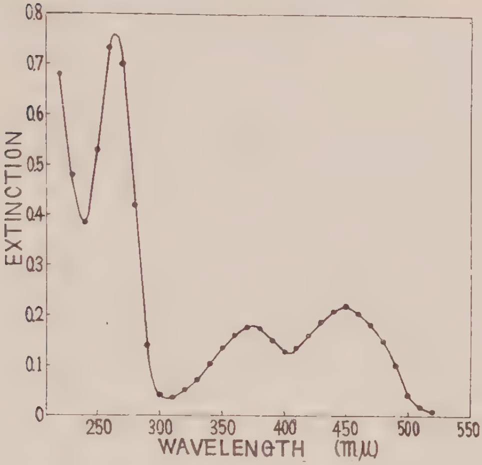

Fig. 1. The absorption spectrum of FAD prepared from *Eremothecium ashbyii*. FAD concentration 1.95×10^{-5} *M* in 0.1 *M* phosphate buffer at pH 7.0.

place, a fact which was already reported by the senior author and others (*13*).

SUMMARY

A large amount of FAD was formed by the culture of *Eremothecium ashbyii*.

FAD contained in this fungus was extracted with warm water in a high yield. The aqueous solution of FAD was concentrated and precipitated by the addition of dehydrated ethanol. The precipitate was extracted with liquid phenol, then the phenolic solution was shaken with ether and water. FAD in the aqueous layer was separated by counter-current distribution method with water and water-saturated benzyl alcohol as the solvents. The FAD fraction was purified by Florisil adsorption chromatography. It was further purified by powdered cellulose chromatography. By these procedures, 47.3 mg. of FAD powder was obtained from 60 g. of wet mycelium containing 102 mg. of FAD. The purity of this sample was 85.3 per cent.

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The Journal of Biochemistry, Vol. 43, No. 1, 1956

NON-UNIFORM LABELING OF THE SILK FIBROIN SYNTHESIZED IN VIVO

Sirs :

Non-uniform labeling of amino acid residues isolated from different positions in the protein molecule has been considered to support the conception of protein formation as a stepwise process (*1–4*). In the present letter we wish to report the results showing non-uniform labeling of the silk fibroin synthesized *in vivo*.

Radioactive glycine labeled with C^{14} on the carboxyl group was injected into the body fluid of the silkworm larva (*Bombyx mori*) at the third to fourth days of the fifth instar. A considerable amount of C^{14}-glycine injected was found to be incorporated into the fibroin molecules of the posterior gland. The dose was 5 mg. glycine per one body (1.2×10^4 c.p.m.). The insects were sacrificed at three and four hours after injection and the posterior glands were removed. The tissue were homogenized with three volumes of 0.14 M NaCl in a glass homogenizer, centrifuged and the supernatant was discarded. The residue was again extracted with the equal volume of 1 M NaCl. The insoluble material was washed several times with distilled water, followed with alcohol and ether as usual manner. The protein thus obtained was subjected to the assy of radioactivity of glycine residues in the different positions of the protein molecule.

Silk fibroin has been shown to contain terminal amino groups derived from glycine, alanine, and serine (*5*). In the present work, therefore, glycine residues were isolated as the 2,4-dinitrophenyl derivative from terminal and non-*N*-terminal positions of the radioactive fibroin by paper-chromatography according to the Sanger's DNP-method (*6, 7*). The comparison between the radioactivities of the terminal and non-N-terminal glycine is shown in Table I, in which the average of all the silk fibroin glycine is also given.

From the data presented, it is evident that, under the conditions of these experiments, the glycine isolated from the amino terminal fraction have a much higher specific activity than the average specific activity of glycine residues in the remaining fraction. These results are not compatible with a mechanism of protein synthesis by means of a complete

TABLE I

Comparison between Radioactivities of N-terminal, Non-N-terminal and Total Glycine Residues in the Radioactive Fibroin of Posterior Silkgland

DNP-glycine	Radioactivity (c.p.m.)	
	Experiment	
	No. 1	No. 2
N-Terminal	477.4±15.7	433.8±23.4
Non-*N*-terminal	52.1± 3.9	43.1± 7.1
Total	51.1± 3.9	69.2± 9.3

1) Each value is expressed in counts per minute (c.p.m.) per 1 μmol. DNP-glycine after correcting for background but for self-adsorption. DNP-glycine was determined with the Beckman model DU spectrophotometer at 345 mμ.

2) Injected 5 mg. C^{14}-glycine (1.2×10^4 c.p.m.) per Silkworm, dissected after 3 hrs. (No. 1) and 4 hrs. (No. 2).

template process (*8*, *9*, *10*). Non-uniform labeling of silk fibroin may be explained by such a mechanism in which a fibroin molecule is synthesized stepwise, involving free or conjugated peptide intermediates. Full details of this work will be published later.

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ON THE PEPTIC MODIFICATION OF CATALASE

Sirs:

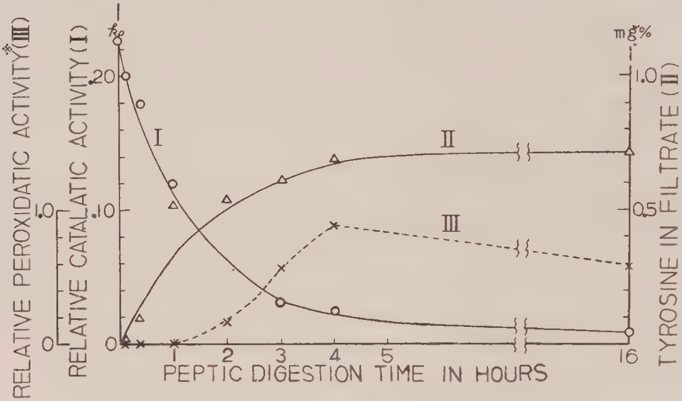
Since Keilin and Hartree (*1*) demonstrated the coupled oxidation of ethanol by catalase and hydrogen peroxide-generating enzyme system, there have appeared many reports on the peroxidatic oxidation of various substances by catalase (*2*). It is, at present, interpreted that hydrogen peroxide and its acceptor (oxidizable substance) compete with each other for the secondary active center on the moleculate of catalase-hydrogen peroxide complex compound. In the previous report (*3*) it has been suggested that the predominant catalatic activity of crystalline beef liver catalase may be attributed, in a greater part, to the specialized structure of its protein moiety, comparing with that of proper peroxidase. Recently, Suzuki *et al.* (*4*) reported on the effects of benzoate or salicylate as a protein perturbator upon the catalatic and peroxidatic activities of purified catalase.

In order to get a clue on the protein structure of catalase, facilitating the predominant catalatic activity, a mild peptic digestion of catalase was carried out at pH 4. The following experimental results were obtained:

1) Prior to peptic digestion, the inactivation of catalase in acid solution varying in pH's values was examined by adding HCl solutions at different concentrations. The acidified solution was neutralized at desired time with an equal volume of respective NaOH solution at correctly corresponding concentration, which followed by the addition of *M*/15 phosphate buffer at pH 7.0. The neutralized solution was diluted to an appropriate volume, an aliquot of which was used for the determination of catalatic activity by permanganate titration. A slow inactivation of catalase occurred at pH 4.8–3.6, and the activity was reduced to 87 per cent in 30 minutes at room temperature. At pH 3.0 the activity was lowered promptly to 71 per cent, and was almost completely lost in 30 minutes. Below pH 2.8 almost perfect inactivation was observed as soon as the catalase solution was acidified.

2) For peptic digestion, the catalase solution was, therefore, incubated with purified pepsin above pH 4.0. Above pH 4.4 the catalase inactivation and proteolysis proceeded quite slowly. At pH 4.0 a fairly progressive decrease in the catalatic activity was observed as shown in Fig. 1, Curve I. On the other hand, the proteolytic process was shown

to be approximately corresponding to the inactivation curve (Fig. 1, Curve II). In this case, however, the grade of proteolysis, which was estimated by Folin-Ciocalteau's phenol reagent with trichloroacetic acid filtrate, was calculated at equilibrium to be about 10 per cent of the complete hydrolysis, while the catalatic activity was mostly lost.



* Purpurogallin formed (mg.)/ml. digest/15 min. at 0°.

$k_0=\frac{1}{t}\log\frac{a_0}{a_0-x}$ per ml. digest.

3) Since this modified catalase molecules are expected to have still intact hematin groups attached to partially digested protein moiety, the peroxidatic activity was examined at intervals during the incubation by Kodama and Masamune's pyrogallol method (*5*). As shown in Fig. 1, Curve III, the peroxidatic activity appeared in 1–2 hours, and it increased with incubation time as long as the catalatic activity was being lowered.

4) For detection of the modified catalase in this way, the digested specimens were pipetted out at intervals for paper chromatographic (butanol-acetic acid-water system) and paper electrophoretic analyses (phosphate buffer at pH 7.0), followed by spraying with BPB and ninhydrin solutions. There was, however, detectable no spot corresponding to such a partially hydrolyzed catalase molecule under the present condition.

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PURIFICATION AND SOME PROPERTIES OF CYTOCHROME *b* FROM OX HEART MUSCLE

Sirs:

As previously reported by Yakushiji and Okunuki (*1*, *2*), sodium cholate was very favourable for extraction of cytochromes *a* and c_1 from ox heart muscle. The 2 per cent cholate extract contained a considerable amount of cytochrome *b*, but the further purification of this component was not possible because of its instability. Therefore, the soluble preparation of cytochrome *b* has been only obtained by Yakushiji and Mori (*3*) from baker's yeast. Recently, Hübscher *et al.* (*4*) and Widmer *et al.* (*5*) have obtained the soluble cytochrome *b* preparation freed from cytochrome *a* by using low concentrations of sodium cholate solution, but these were also unstable and still contained other components detectable spectrophotometrically. In the processes of further purification of cytochromes *a* and c_1, authors succeeded to obtain the highly purified cytochrome *b* and the complex of cytochromes *b* and c_1 in a crystalline form from cholate solution.

The cholate extract of ox heart was fractionated with ammonium sulfate as treated in the case of preparation of cytochromes *a* and c_1 (*1*, *2*). In these ways, the component *b* was led to insoluble state and thus separated from cytochrome *a*. This insoluble fraction was collected and suspended in cholate solution (pH 7.6) and digested with a small amount of crystalline bacterial proteinase prepared from the culture medium of *Bac. subtilis* according to the method of Hagihara (*6*). After the digestion this precipitate became again soluble, and the highly purified cytochrome *b* was obtainable in a crystalline form by successive fractionations with ammonium sulfate.

This cytochrome *b* preparation was deep reddish in solution like hemoglobin, very stable in cold for a month, and autoxidizable, and did not combine with cyanide. As shown in Fig. 1, the absorption bands were found at 280 and 416 mμ in oxidized state, at 430, 530, and 564 mμ after reduction with sodium dithionite, and at 426, 528, and 558 mμ by addition of pyridine. The oxidation-reduction potential measured by electric titration method was -50 mV., so the component was not reduced by ascorbic acid. Furthermore, since the component *b* was not reduced by succinate or by reduced diphosphopyridine nucleotide in the presence of diaphorase (*7*), cytochrome *b* was concluded to be not

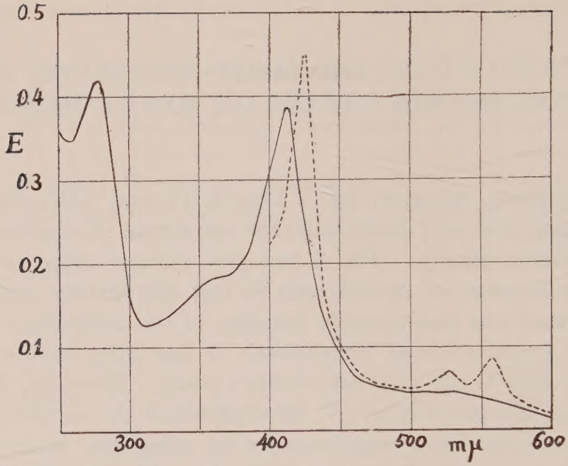
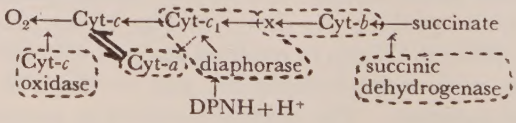


Fig. 1. Absorption spectra of cytochrome *b*.
Solid line; oxidized form, Dotted line; reduced form.

identical with succinic dehydrogenase and not to link with diaphorase.

In spite of the differences of the saturation degrees of ammonium sulfate for each fraction of cytochrome *b* and cytochrome c_1, it was not succeeded to separate these two components from the complex preparation of cytochromes *b* and c_1 prepared by autolysis of the first ammonium sulfate fraction of the cholate extract, but above two components were able to separate after digestion with bacterial proteinase. The complex of cytochromes *b* and c_1 also showed a fairly strong activity of diaphorase and the α-band of cytochrome c_1 at 552 mμ was reduced by additions of reduced diphophopyridine necleotide, but cytochrome *b* was not reduced. Considering above results in connection with the one of Yakushiji and Okunuki (*1*, *2*), it can be concluded that the electron transfer of reduced diphosphopyridine nucleotide occurs as follows :



where enclosure with dotted line indicates the components extracted in bound form.

The property of cytochrome *b* and the relations between cytochrome *b* and c_1 and diaphorase will be published in details elsewhere.

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